

**APPLICATION AND MODE OF ACTION OF
THE POLY- β -HYDROXYBUTYRATE (PHB)
IN *Penaeus* CULTURE**

GLADYS LUDEVESE PASCUAL

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in
Applied Biological Sciences

Illustration on the cover:

PHB-based biodegradable plastic as artificial substratum for the whiteleg shrimp *Litopenaeus vannamei* postlarvae.

To cite this work:

Ludevese-Pascual G. (2016) Application and mode of action of the poly- β -hydroxybutyrate in *Penaeus* culture. PhD thesis, Ghent University, Belgium.

This work was funded by the VLIR-UOS (Flemish Interuniversity Council), Brussels Belgium, through the ICP PhD Sandwich Programme doctoral grant awarded to Gladys Ludevese-Pascual.

The author and promoters give the authorization to consult and to carry parts of this work for personal use only. Permission to reproduce any material contained in this work should be obtained from the author.

Members of the examination and reading committee (*):

Prof. dr. ir. Peter Bossier (Flemish promoter)
Department of Animal Production
Ghent University, Belgium

Dr. ir. Peter De Schryver (Flemish co-promoter)
Department of Animal Production
Ghent University, Belgium

Present address:
INVE Technologies
Dendermonde, Belgium

Dr. Edgar Amar (Local promoter)
Fish Health Section
Southeast Asian Fisheries Development Center/Aquaculture Department
Iloilo Philippines

Prof. Em. Patrick Sorgeloos*
Department of Animal Production
Ghent University, Belgium

Dr. ir. Marc Verdegem*
Aquaculture and Fisheries Group (AFI)
Wageningen University, The Netherlands

Prof. dr. ir. Mieke Uyttendaele*
Department of Food Safety and Food Quality
Ghent University, Belgium

Prof. dr. ir. Geert Janssens*
Department of Nutrition, Genetics and Ethology
Ghent University, Belgium

Prof. dr. ir. Monica Höfte (Chairwoman)
Department of Crop Protection
Ghent University, Belgium

*“Now unto him that is able to do exceeding abundantly above all that we ask or think, according to the power that worketh in us...Unto **HIM** be glory”*

Ephesians 3:20-21

LIST OF ABBREVIATIONS

ADG	Average daily growth
ANOVA	Analysis of variance
β -HB	β -hydroxybutyric acid and/or its conjugate base β -hydroxybutyrate
BW	Body weight
CDW	Cell dry weight
DAH	Day(s) after hatching
DHA	Docosahexaenoic acid
EPA	Eicosapentanoic acid
IRMS	Isotope Ratio Mass Spectrometer
FAs	Fatty acids
FASW	Filtered autoclaved seawater
GC-c-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GI	Gastrointestinal tract
HUFAs	Highly unsaturated fatty acids
KB	Ketone bodies
LCFAs	Long-chain fatty acids
LC ₅₀	Lethal concentration required to kill 50 % of the population
LNA	Linolenic acid
LOA	Linoleic acid
LSI	Larval stage index
NH ₄ ⁺ -N	Ammonium-nitrogen
NH ₃ ⁻ -N	Ammonia-nitrogen
PHB	Poly- β -hydroxybutyrate
PHAs	Polyhydroxyalkanoates
PL	Postlarvae
PUFAs	Poly-unsaturated fatty acids
PVC	Polyvinylchloride
SCFAs	Short-chain fatty acids
SELCO	Self-emulsifying lipid concentrate
SEM	Standard error of mean
TAN	Total ammonia-nitrogen
TIN	Total inorganic nitrogen
TL	Total lipid
VPDB	Vienna Pee Dee Belemnite

CONTENTS

List of abbreviations	9
Contents	10
List of Tables	12
List of Figures	15
Preface	19
Chapter 1	Literature review and objectives of the study
1.1	Feeding the World through Aquaculture
1.2	Penaeid shrimp aquaculture
1.3	Penaeid shrimp nutrition
1.4	Diseases as major constraint in penaeid shrimp culture
1.5	Shrimp disease management strategies
1.6	Poly- β -hydroxybutyrate (PHB): A new strategy for sustainable aquaculture
1.7	Rationale and objectives of the study
Chapter 2	Poly- β -hydroxybutyrate (PHB) enriched <i>Artemia</i> sp. for tiger shrimp <i>Penaeus monodon</i> larviculture
	Abstract
2.1	Introduction
2.2	Materials and Methods
2.3	Results
2.4	Discussion
	Acknowledgment
Chapter 3	The effects of poly- β -hydroxybutyrate (PHB) supplementation on the lipid and fatty acid composition of crustaceans: <i>Artemia</i> sp. as model species
	Abstract
3.1	Introduction
3.2	Materials and Methods
3.3	Results
3.4	Discussion
	Acknowledgement
Chapter 4	Assessment of poly- β -hydroxybutyrate (PHB) assimilation by crustaceans using stable ^{13}C isotope tracing
	Abstract
4.1	Introduction
4.2	Methodology
4.3	Results
4.4	Discussion
	Acknowledgment

Chapter 5	Application of poly- β -hydroxybutyrate (PHB)-based bioplastics as artificial substratum in penaeid shrimp culture	
	Abstract	160
5.1	Introduction	162
5.2	Materials and Methods	166
5.3	Results	177
5.4	Discussion	193
	Acknowledgement	199
Chapter 6	Conclusions and Future perspectives	
6.1	Introduction	202
6.2	Main accomplishments	202
6.3	Disease management in aquaculture	203
6.4	The mode of action of poly- β -hydroxybutyrate: Host	206
6.5	The mode of action of poly- β -hydroxybutyrate: Pathogen	214
6.6	The mode of action of poly- β -hydroxybutyrate: Environment	216
6.7	Some limitations on the application of PHB as substratum in penaeid shrimp culture	219
6.8	General conclusions	221
References		229
Summary		287
Samenvatting		291
Appendix 1: Curriculum vitae		297
Appendix 2: Acknowledgement		303

LIST OF TABLES

Table 1.1	Farming practices for extensive, semi-intensive and intensive shrimp aquaculture (Rönnbäck 2001).	25
Table 1. 2	Example of a feeding schedule applied in Vietnam for intensive culture (20-50 shrimp m ⁻²) of tiger shrimp using formulated feeds (Hung & Quy 2007).	27
Table 1.3	Recommended dietary EFAs in various penaeid species (Perez-Velasquez <i>et al.</i> 2004).	31
Table 1. 4	Protein requirements for various species of penaeid shrimp.	33
Table 1.5	Common pathogens of penaeid shrimp (Lightner <i>et al.</i> 2009).	36
Table 1.6	OIE (Office International des Epizooties)-listed penaeid shrimp diseases as of 2006 and those being considered for listing (Lightner 2011).	37
Table 1.7	Estimated economic losses since the emergence of viral diseases in penaeid shrimp (Lightner 2011).	38
Table 1.8	LC ₅₀ values (48 h) of <i>Vibrio</i> spp. isolated from diseased shrimp (Jayasree <i>et al.</i> 2006).	40
Table 1.9	Commercially available immunostimulants (Apines-Amar & Amar 2015).	48
Table 1.10	PHB (or PHA in general) degrading microorganisms isolated from various environments (Khanna & Srivastava 2005).	59
Table 1.11	Overview of published studies on the application of PHB in various aquatic farmed species.	65
Table 2.1	Weight (mg) and survival (%) of <i>P. monodon</i> postlarvae fed with differentially enriched <i>Artemia</i> after 21 days of feeding. <i>P</i> -value in bold indicates significant difference (<i>P</i> < 0.05).	92
Table 3.1	Effects of supplying dietary PHB either in crystalline or amorphous form on the total lipid content (% on DW) and the content of the four essential FAs in <i>Artemia</i> expressed either on dry weight (mg g ⁻¹ DW) or on total lipid (mg g ⁻¹ lipid; in parenthesis). Values are means ± SEM. Different superscript letters in a column indicate significant difference	

	($P \leq 0.05$; P -values in bold).	114
Table 3.2	Effects of co-supplying PHB in crystalline or amorphous form with the bacterial PHB degrader <i>C. testosteroni</i> on the total lipid content (% on DW) and the content of the four essential FAs in <i>Artemia</i> expressed either on dry weight (mg g ⁻¹ DW) or on total lipid (mg g ⁻¹ lipid; in parenthesis). Values are means \pm SEM. Different superscript letters in a column indicate significant difference ($P \leq 0.05$; P -values in bold).	116
Table 3.3	Effects of co-supplying PHB in crystalline to HUFA-rich lipid emulsion on the total lipid content (% on DW) and the content of the four essential FAs in <i>Artemia</i> expressed either on dry weight (mg g ⁻¹ DW) or on total lipid (mg g ⁻¹ lipid; in parenthesis). Values are means \pm SEM. P -values in bold indicate significant differences ($P \leq 0.05$; P -values in bold).	118
Table 4.1.	Dietary feed composition.	134
Table 4.2.	$\delta^{13}\text{C}$ values with their corresponding contents (in area %) in diet either supplemented with non-labeled <i>R. eutropha</i> DSM545 or ^{13}C -labeled <i>R. eutropha</i> DSM545. Values obtained from two replicates (separated by semi-colon).	143
Table 4.3.	Mean $\delta^{13}\text{C}$ values (‰ \pm SEM) for fraction of neutral lipid extracted from degutted postlarval whiteleg shrimp ($n = 3$ or 4). Different letter superscript indicates significant difference ($P \leq 0.05$).	145
Table 4.4.	Mean $\delta^{13}\text{C}$ values (‰ \pm SEM) for fraction of glycolipids extracted from degutted postlarval whiteleg shrimp ($n = 3$ or 4). Different letter superscript indicates significant difference ($P \leq 0.05$).	146
Table 4.5.	Mean $\delta^{13}\text{C}$ values (‰ \pm SEM) for fraction of phospholipids extracted from degutted postlarval whiteleg shrimp ($n = 3$ or 4). Different letter superscript indicates significant differences ($P \leq 0.05$).	147
Table 4.6.	^{13}C enriched-FAs* with their corresponding contents (in area %) in various lipid fractions extracted from degutted postlarval whiteleg shrimp. Values are means (either having 3 or 4 replicates) \pm SEM.	148
Table 4.7.	Comparison between ^{13}C enrichment in diet supplemented with ^{13}C -labeled <i>R. eutropha</i> DSM545 and in various lipid fractions of degutted postlarval whiteleg shrimp.	151

Table 5.1	Dietary feed composition.	167
Table 5.2.	Survival (%) and growth of postlarval tiger shrimp provided either PVC or PHB substratum in the culture tank. Values represent means \pm SEM ($n = 5$). Different letters within a row indicate significant differences ($P \leq 0.05$; P -value in bold).	178
Table 5.3.	Whole-body lipid content (% on DW) and content of four essential FAs expressed either on dry weight (mg g^{-1} DW) or on total lipid (mg g^{-1} lipid; in parenthesis) of postlarval tiger shrimp cultured for 61 days in the presence of either PVC substratum or PHB substratum. Values are means \pm SEM. Different superscript letters in a column indicate significant difference ($P \leq 0.05$; P -values in bold).	184
Table 5.4.	Survival (%) and growth of postlarval whiteleg shrimp provided either PVC or PHB substratum in the culture tank. Values represent means \pm SEM ($n = 7$). Different letters within a row indicate significant differences ($P \leq 0.05$; P -value in bold).	185

LIST OF FIGURES

Figure 1.1	Relative contribution of aquaculture and capture fisheries to food fish consumption (FAO 2014a).	23
Figure 1.2	Generalized feeding scheme for penaeid shrimp from zoea to late PL stages (modified from Kungvankij <i>et al.</i> 1985). E = egg; N = nauplius; PZ = protozoa; My = mysis; PL = postlarvae.	27
Figure 1.3	Hydrolysis of lipids.	30
Figure 1.4	Relative economic loss in 2001 related to disease caused by various pathogen groups (Flegel <i>et al.</i> 2008).	36
Figure 1.5	Transmission electron microscopy observation of <i>Azotobacter chroococcum</i> . 0% PHB content (A) and 85 % PHB content (B) (Bonartsev <i>et al.</i> 2007)	56
Figure 1.6	Scheme of a PHB granule (Zinn <i>et al.</i> 2001).	56
Figure 1.7	Utilization of β -HB and AcAc in the mammalian brain during prolonged starvation (Laffel 1999).	72
Figure 1.8	Mechanism of antipathogenic activity of SCFAs (taking butyric acid as example). The fatty acids pass the cell membrane in their undissociated form and dissociate in the cytoplasm. As a consequence, the cells have to spend energy to export excess protons (Defoirdt <i>et al.</i> 2009).	77
Figure 2.1	Larval stage index (LSI) of <i>P. monodon</i> postlarvae fed non-enriched (Art - P - L) and enriched <i>Artemia</i> (lipid emulsion-enriched (Art - P + L); PHB-enriched (Art + P - L); and PHB + lipid-emulsion enriched (Art + P + L)) during the 21 day feeding trial. Values represent means \pm SEM ($n = 4$). No significant differences were detected.	93
Figure 2.2	Survival (%) of <i>P. monodon</i> postlarvae fed differentially enriched <i>Artemia</i> for 21 days and subsequently exposed for 24 h to a lethal dose of ammonium chloride. Bars represent means \pm SEM ($n = 4$). Bars with different letters are significantly different ($P \leq 0.05$). Interaction between PHB and lipid was statistically significant ($F = 5.261$, $P = .043$) by which the PHB effect was nullified in combination with lipid enrichment.	94
Figure 2.3	Cumulative mortality (%) of <i>P. monodon</i> postlarvae fed non-enriched (Art - P - L) and enriched <i>Artemia</i> (lipid emulsion-enriched (Art - P + L); PHB-enriched (Art + P - L); and PHB + lipid-emulsion enriched (Art + P + L)) followed by exposure to a sublethal dose of ammonium chloride for 24 h and	

	subsequent challenge with pathogenic <i>V. campbellii</i> LMG21363. Values represent means \pm SEM ($n = 4$). Statistical analysis done at 15 days after challenge showed a significant interaction between PHB and lipid ($F = 9.597$, $P = .015$). PHB effect was nullified when combined with lipid enrichment.	96
Figure 4.1	Mean $\delta^{13}\text{C}$ of <i>Artemia</i> nauplii supplied different dietary treatments (Art + <i>T. suecica</i> (D1); Art + ^{13}C -labeled <i>R. eutropha</i> DSM545 (D2); Art + <i>T. suecica</i> + ^{13}C -labeled <i>R. eutropha</i> DSM545 (D3); Art + LVS3 + ^{13}C -labeled <i>R. eutropha</i> DSM545 (D4); Art + <i>D. tertiolecta</i> + ^{13}C -labeled <i>R. eutropha</i> DSM545 (D5)) in 48 h. Values are presented as mean \pm SEM ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$).	138
Figure 4.2	Mean $\delta^{13}\text{C}$ of <i>Artemia</i> nauplii in function of time. Values are presented as mean \pm SEM ($n = 3$). Different letters indicate significant difference from time 0 ($P \leq 0.05$).	139
Figure 4.3	Mean $\delta^{13}\text{C}$ of postlarval whiteleg shrimp fed ^{13}C -labeled <i>R. eutropha</i> DSM545 supplemented either in sub- or optimal diets. Values represent mean \pm SEM ($n = 4$). Asterisk indicates significant difference between dietary treatments within a time point ($P \leq 0.05$)	141
Figure 5.1	Weight frequency distribution of all surviving postlarval tiger shrimp provided either PVC (1) or PHB (2) substratum in the culture tank.	179
Figure 5.2	Proportion of surviving postlarval tiger shrimp cultured with either PVC or PHB substratum and subsequently exposed for 99 h to a lethal dose of ammonium chloride at 143 mg L^{-1} with a mean pH of 8.2. Values represent means \pm SEM ($n = 5$).	180
Figure 5.3	Proportion of surviving postlarval tiger shrimp cultured with either PVC or PHB substratum followed by exposure to a sub-lethal dose of ammonium chloride for 24 h and subsequent challenge with pathogenic <i>V. campbellii</i> LMG 21363. Values represent means \pm SEM ($n = 5$).	181
Figure 5.4	Substratum visit (%) by postlarval tiger shrimp on PVC and PHB substrata. Data are represented as means \pm SEM ($n = 5$). Asterisk indicates significant difference between treatments within a time point as indicated with a box ($P \leq 0.05$).	182
Figure 5.5	Weight frequency distribution of all surviving postlarval whiteleg shrimp provided either PVC (1) or PHB (2) substratum in the culture tank.	186
Figure 5.6	Faecal matter collected from postlarval whiteleg shrimp cultured in the presence of either PVC substratum or PHB substratum.	187

Figure 5.7	Visit and preference by postlarval whiteleg shrimp on PVC and PHB substrata. Values are represented as means \pm SEM ($n = 5$). Asterisk indicates significant difference between treatments within a time point as indicated with a box ($P \leq 0.05$).	188
Figure 5.8	Changes in the levels of the total ammonia nitrogen (TAN) ($\text{NH}_3\text{-N} + \text{NH}_4^+\text{-N}$; panel A), nitrite-nitrogen ($\text{NO}_2\text{-N}$; panel B), nitrate-nitrogen ($\text{NO}_3\text{-N}$; panel C) and their sum (panel D) in tanks provided with no substratum (control), PVC substratum or PHB substratum in the presence of an initial concentration of 1.75 mg L^{-1} TAN. Data are means \pm SEM ($n = 3$). Different letters indicate significant difference among treatments at a specific time point ($P \leq 0.05$).	190
Figure 5.9	Changes in the levels of the total ammonia nitrogen (TAN) ($\text{NH}_3\text{-N} + \text{NH}_4^+\text{-N}$; panel A), nitrite-nitrogen ($\text{NO}_2\text{-N}$; panel B), nitrate-nitrogen ($\text{NO}_3\text{-N}$; panel C) and their sum (panel D) in tanks provided no substratum (control), PVC substratum or PHB substratum in the presence of whiteleg shrimp postlarvae at a density of 1 individual per L^{-1} . Data are means \pm SEM ($n = 3$). Different letters indicate significant difference among treatments at a specific time point ($P \leq 0.05$).	192
Figure 6.1	Interactive factors influencing disease in aquaculture (redrawn from Nemutanzhela <i>et al.</i> 2014).	205
Figure 6.2	PHB-based bioplastic type P209: original material (A); material immersed in a recirculating tank of the Shrimp Facility of ARC for 15 days (B).	220
Figure 6.3	Figure 6.2. Contribution of PHB in penaeid shrimp	222
Figure 6.4	Proposed lines of studies pertaining to the utilization of dietary PHB as alternative sources of energy in the absence of carbohydrates and/or protein as sources of energy in penaeid shrimp	224

“Shrimp is the fruit of the sea. You can barbecue it, boil it, broil it, bake it, sautee it. There's, um, shrimp kebabs, shrimp creole, shrimp gumbo, pan fried, deep fried, stir fried. There's pineapple shrimp and lemon shrimp, coconut shrimp, pepper shrimp, shrimp soup, shrimp stew, shrimp salad, shrimp and potatoes, shrimp burger, shrimp sandwich... That's, that's about it.”

-Mykelti Williams

Preface

The depletion of wild fish stock gives impetus for the rapid growth of aquaculture to be able to comply with the demands of the ever increasing human population (Tidwell & Allan 2001). As human population is projected to reach 9.3 billion by 2050 (UN 2010), it is anticipated that aquaculture will need to be the main source of fish supply.

With the continuing increase of aquaculture output, concerns have risen about its impact on the environment. Losses of mangrove forests, biological pollution as a consequence of the introduction of exotic species are only a few of the documented impacts of aquaculture. Aquaculture is also widely recognized as an important cause for the introduction, transfer and spread of aquatic diseases (Walker & Winton 2010). It is well documented that the outbreaks of diseases in aquaculture do not only hamper significant increase in production, but also jeopardize the ecological integrity and productivity of adjacent coastal waters (Martinez-Porchas & Martinez-Cordova 2012). For these reasons, several efforts have been made to prevent diseases in aquaculture or at least mitigate their impacts. Common strategies range from disinfection of the rearing water to application of chemotherapy (e.g. antibiotics). However, practices such as the application of antibiotics, are increasingly becoming undesirable as it promote the selection for antibiotic-resistance in both the target pathogenic bacteria, as well as in other microorganisms present in the environment.

Recently, an alternative approach was suggested implying the use of the bacterial storage compound poly- β -hydroxybutyrate (PHB). PHB is a naturally occurring polyester that is produced and accumulated by microorganisms mainly under conditions of nutrient

limitation and carbon excess (Reddy *et al.* 2003). PHB is hypothesized to depolymerize into β -HB monomer that is known to exhibit antimicrobial, insecticidal, and antiviral activities comparable to other short chain fatty acids (SCFAs) (Tokiwa & Ugwu 2007; Defoirdt *et al.* 2006). In addition, PHB and/or its degradation product β -HB can probably also be used as an energy source (De Schryver *et al.* 2010b). The first known application of PHB in aquaculture was reported by Defoirdt *et al.* (2007b) and this was consequently tested in various aquatic farmed species. Improved survival, growth promotion, enhanced resistance against pathogens and improved overall performance of the tested aquatic farmed species were reported (De Schryver *et al.* 2010a; Defoirdt *et al.* 2007b; Nhan *et al.* 2010; Sui *et al.* 2012; Suguna *et al.* 2014; Halet *et al.* 2007; Laranja *et al.* 2014; Thai *et al.* 2014). Recently, PHB has been shown to affect lipid metabolism in fish (Najdegerami *et al.* 2015) and it is distributed in different organs following digestion and absorption (Situmorang *et al.* 2015). Despite the reports on the beneficial effects of PHB in various aquatic farmed species, its actual mechanisms of action remained largely unexplored. There are also very few attempts made to explore the application of PHB in penaeid shrimp being the most important internationally traded fishery commodity in terms of value and the most valuable fishery export for many tropical developing countries (FAO 2009; Bondad-Reantaso *et al.* 2012).

The ability of the aquaculture sector to foresee and react to the trends in demand for and consumption of fish is of vital importance to the future development and success of this sector. Indeed, the road to sustainable aquaculture requires collective and concerted efforts of all the actors involved.

Chapter 1

Literature review and objectives of the study

1.1 Feeding the world through aquaculture

Fish is a vital food for people (Tidwell & Allan 2001). Studies on human nutrition and physiology provide fundamental evidence on fish consumption by early humans (Erlandson 2001). It was once generally thought that the natural food reserves in oceans were limitless and could provide enough food to meet the need of the human population (Drakeford & Pascoe 2008). However, as the human population has been growing continuously, the demands for fish food has also increased resulting in wild stocks being fished to capacity (FAO 2002*a*). In addition, factors such as destructive fishing practices have also contributed to the decline of fish stocks (Rebufat 2007). The collapse of the large, profitable fisheries such as the Californian sardine in the 1950s, the Atlanto-Scandian herring in the late 1960s, the Peruvian anchovy in 1972, the Northern cod off the East coast of Canada in 1992 and the North Sea cod have acted as clear warnings that fishing practices in many parts of the world are not sustainable and that there are serious issues with the management of natural fish stocks (Hauge *et al.* 2009).

Aquaculture is the farming of fish, crustaceans, bivalves and aquatic plants both inland and in coastal areas involving human interventions in the rearing process to enhance production (FAO 2000). This sector is believed to be able to replace capture fisheries as the primary source of food fish as shown in Figure 1.1 (FAO 2014*a*). It has been reported that aquaculture production increased at an average annual rate of 6.2 % in the period 2000–2012 (from 32.4 million to 66.6 million tonnes).

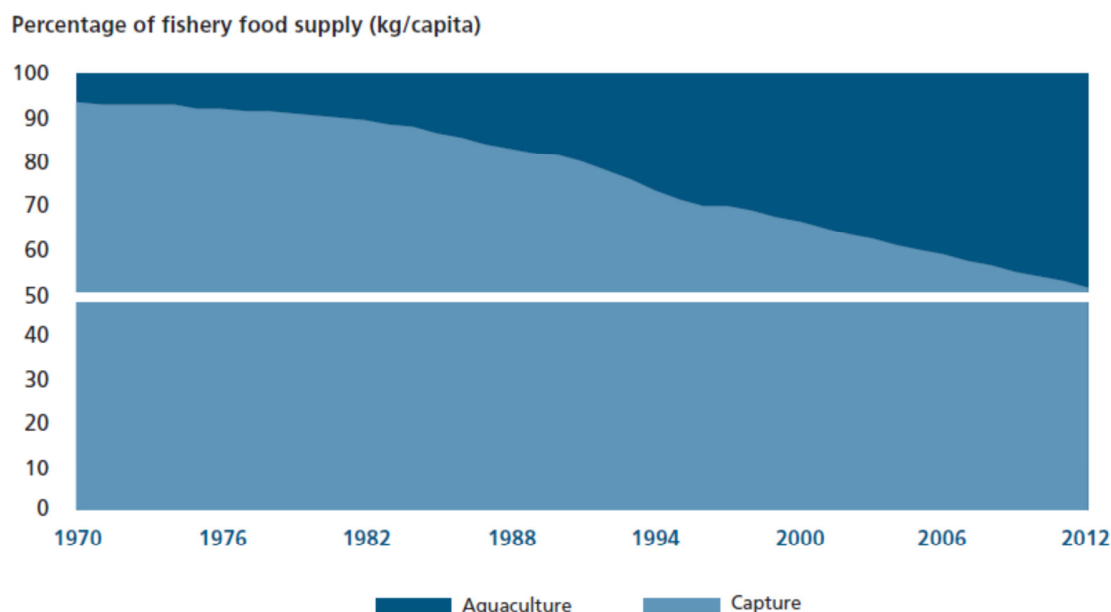


Figure 1.1 Relative contribution of aquaculture and capture fisheries to food fish consumption (FAO 2014a).

By 2030, global aquaculture production is estimated to reach approximately 118 million tonnes, which is more than three times higher than the output of 2000 (FAO 2014a). As human population is projected to reach 9.3 billion by 2050 (UN 2010), it is anticipated that aquaculture will act as the main source of supply of fish food, as less than half of seafood consumed will come from fishing catches.

1.2 Penaeid shrimp aquaculture

One of the most significant changes in marine and coastal environments since the mid-twentieth century has been the uprise of coastal shrimp aquaculture (Pokrant 2014). Although the history of penaeid shrimp culture is not well-documented, its origin traces back to the earliest brackish water and marine ponds. These primitive ponds, which date back thousands of years in the Mediterranean area (Brown 1983), and to the 15th century A.D. in Indonesia were typically stocked with fish, shrimp and other biota that

grew through passive feeding (Ling 1977). In Asia, early methods of shrimp culture were either through low-density monocultures or polyculture with fish. Another system widely practised in West Bengal and in other parts of India was the rotation of rice and shrimp in the same field (Shiva & Karir 1997).

Until the mid-1970s, shrimp culture heavily relied on shrimp seeds collected from gravid females taken from the wild. However, this strategy became problematic for the reason that gravid females are not consistently available (Rönnbäck 2001). The first successful captive spawning of mature Kuruma shrimp *Penaeus japonicus* performed by Japanese biologists in 1933 was a major breakthrough in penaeid shrimp aquaculture. The discovery of eyestalk ablation or enucleation in the early 1970s was also a significant success in the history of penaeid shrimp aquaculture. This technique employs the removal of the eyestalk ganglia containing the neurosecretory organs that secrete, store and release the gonad inhibiting hormone (GIH) in order to stimulate rapid ovarian maturation (Treerattrakool *et al.* 2008). The improved knowledge on reproduction combined with the introduction of commercially formulated feeds and the high profits from cultured shrimp contributed to the significant expansion of the shrimp industry (Fast & Menasveta 2000).

The culture of shrimp basically involves a two-step process: a broodstock-hatchery phase for producing seed up to postlarvae and a grow-out phase that is usually performed in earthen culture ponds for on-growing of fry to marketable size (Primavera 1994). Primary management systems used in penaeid shrimp farming in the Southeast Asia can be categorized as extensive, semi-intensive and intensive as shown in Table 1.1.

Table 1.1 Farming practices for extensive, semi-intensive and intensive shrimp aquaculture
(Rönnbäck 2001).

	Extensive	Semi-intensive	Intensive
Pond size (ha)	1-10	1-2	0.1-1
Stocking	Natural + artificial	Artificial	Artificial
Stocking density (seed m ⁻²)	1-3	3-10	10-50
Seed source	Wild + Hatchery	Hatchery + wild	Hatchery
Annual production (tons ha ⁻¹ yr ⁻¹)	0.6-1.5	2-6	7-15
Feed source	Natural	Natural + Formulated	Formulated
Fertilisers	Yes	Yes	Yes
Water exchange	Tidal + pumping (< 5 % daily)	Pumping (< 25 % daily)	Pumping (> 30 % daily)
Aeration	No	Yes	Yes
Diversity of crops	Majority monoculture, some polyculture with fish	Monoculture	Monoculture
Disease problems	Rare	Moderate to frequent	Frequent
Employment (persons ha ⁻¹)	< 7	1-3	1
Production cost kg ⁻¹ (US \$)	1-3	2-6	4-8

Super-intensive systems and recirculating aquaculture systems (RAS) have also been introduced. The characteristics of these farming systems are described in details elsewhere (Chamberlain 2010; Recirculating Farms Coalition 2013).

Farmed shrimp contributes to 54 % of the world's total supply of shrimp in 2011 (FAO 2013a) and climbed to 56 % in 2013 (FAO 2015). It also emerged as one of the most important internationally traded fishery commodity in terms of value and the most valuable fishery export for many tropical developing countries (FAO 2009; Bondad-Reantaso *et al.* 2012). In 2014, about 78 % of the total import volume to the U.S. came from the following five countries: India, Indonesia, Ecuador, Vietnam, and Thailand (Department of Commerce-Bureau of the Census 2015). In Asia, the tiger shrimp *P. monodon* was once the dominating penaeid shrimp cultured (Rosenberry 1999), however, production volume of *Litopenaeus vannamei* (formerly *P. vannamei*) overtook *P. monodon* between 2003-2004 (Larkin 2012).

1.3 Penaeid shrimp nutrition

Penaeid shrimp is known to consume a variety of food items. Under natural conditions, they are regarded as omnivorous scavengers or detritus feeders (Varadharajan & Pushparajan 2013). Penaeid shrimp starts feeding at protozoa stage and the diet ranges from phytoplankton at the early mysis stage to zooplankton such as rotifers, copepods, etc. at the early postlarval (PL) stage. As larvae grow older than PL6, feeding habit changes to that of a bottom feeder. At farm level, penaeid shrimp older than PL6 are given polychaetes, chopped mussel, cockle or bivalve meat and shift to formulated feeds as shrimp grows older than PL20-21 (Figure 1.2).

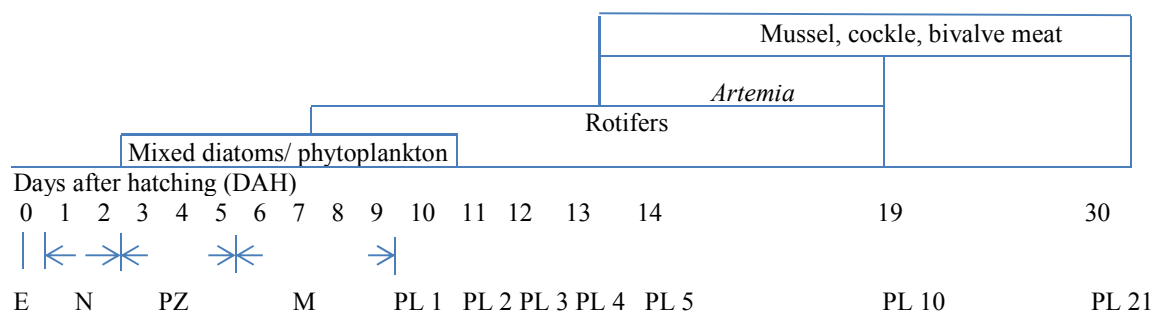


Figure 1.2 Generalized feeding scheme for penaeid shrimp from zoea to late PL stages (modified from Kungvankij *et al.* 1986). E = egg; N = nauplius; PZ = protozoa; My = mysis; PL = postlarvae.

In semi-intensive shrimp culture, formulated feeds are only given when the natural feeds are almost exhausted while in intensive shrimp culture, formulated feeds are given either two, three or four times a day at a feeding rate ranging from 3-10 % of wet body weight (BW) (Table 1.2) (Hung & Quy 2013). Penaeid shrimp requirement for energy sources (i.e. carbohydrates and lipids) and protein is discussed in the succeeding sections.

Table 1.2 Example of a feeding schedule applied in Vietnam for intensive culture (20-50 shrimp m⁻²) of tiger shrimp using formulated feeds (Hung & Quy 2013).

Size (grams)	Feeding (frequency/day)	Feeding rate (% wet BW)	Feed type (diameter; length) (cm)
PL25-4	4	7-8	1.2; 2-4 (starter)
5-8	3-4	5-6	1.4; 2-4 (grower)
9-102	3	3-4	1.7; 2.4 (finisher)

1.3.1 Energy requirement

1.3.1.1 Carbohydrates

Commercial diets are usually formulated to contain adequate amounts of non-protein energy in the form of carbohydrates. Although carbohydrates are not essential, they are considered as the most economical and inexpensive source of energy with protein-sparing and lipid-sparing effects (Davis 2005). Carbohydrates are classified between simple (glucose, trehalose) and complex sugars (starch, glycogen, chitin, cellulose) (Cuzon *et al.* 2000). In general, complex sugars such as starch and dextrin are the most common examples of polysaccharides used in shrimp diets (Rosas *et al.* 2000). Shiau & Peng (1992) have shown that giant tiger shrimp juveniles (0.54 ± 0.02 g) fed diet supplemented either starch or dextrin have significantly higher final weight (2.86 or 2.73 g), feed efficiency ratio (FER) (0.50 or 0.47) and survival (64 or 65 %) than juveniles fed diet supplemented with glucose (final weight, FER and survival of 1.57 g, 0.38 and 56 %, respectively). The mechanism responsible for the poor utilization of simple sugars such as dietary glucose by penaeid shrimp is not yet fully understood but possible explanation could be accounted to glucose saturation due to its high rate of absorption across the digestive tract (Shiau 1998). The complex carbohydrates such as starch undergo enzyme hydrolysis such as conversion to glucose in the midgut and finally, conversion into trehalose in the hepatopancreas before their final release in the blood. Thus, glucose originating from the enzymatic hydrolysis of starch appears slower at gut absorption sites than free glucose that is readily absorbed from the diet (Iba *et al.* 2014).

There is no absolute dietary carbohydrate requirement established for penaeid shrimp but supplementation in sufficient amounts is recommended as they also act as excellent binders in diet formulation producing water stable and palatable diets (Cuzon *et al.* 2000).

1.3.1.2 Lipids

Lipids are important nutrients for penaeid shrimp as they form components of cell membranes and act as energy reserves (FAO 2014b). Shivaram & Raj (1997) mentioned that the dietary lipid requirement in penaeid shrimp diets should range from 5-12 % on dry weight (DW). Overall, the dietary lipids required by penaeid shrimp can be categorized into classes of neutral lipids (including essential fatty acids (EFAs), sterols, phospholipids and glycolipids (Tocher & Glencross 2015). Neutral lipids are primarily responsible for storage of energy in the form of triacylglycerols (TAG) and other storage components such as steryl esters (Tansey *et al.* 2001) while sterols and phospholipids are essential for optimal survival and growth.

The digestion of dietary lipids occurs in the pyloric region of the crustacean stomach. The major digestive enzyme that acts upon lipids in the gastric juice is lipase which hydrolyzes the esteric bonds of TAG. Lipases are liberated by B cells of the midgut gland into the tubule lumen where they move into the gastric chamber and initiate lipid digestion of ingested food. Digested lipid is finally absorbed into the depot-lipid in the form of an ester of glycerol with fatty acid as shown in Figure 1.3 (Rivera-Pérez *et al.* 2010).

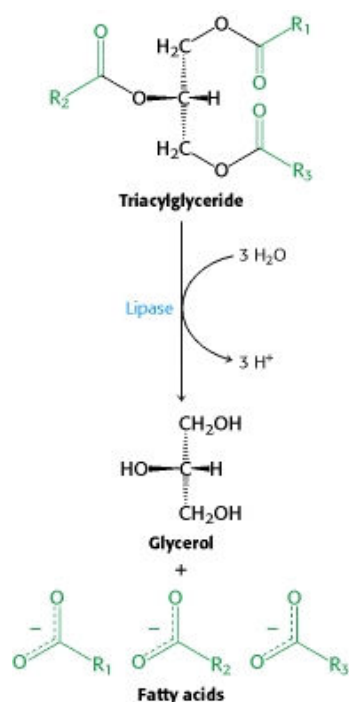


Figure 1.3 Hydrolysis of lipids.

EFA's are important for the maintenance and integrity of cellular membranes as they are integral constituents of phospholipids, which are the fundamental component of the lipid bilayer of cellular membranes (Naik 2010). In particular, penaeid shrimp has a dietary requirement for polyunsaturated fatty acids (PUFAs) namely, linoleic acid (LOA; 18:2 (n-6)) and linolenic acid (LNA; 18:3 (n-3)). LOA has the first double bond between the 6th and 7th carbon atoms from the methyl end and has the greatest EFA value for homothermic animals while LNA has the first double bond between the 3rd and 4th carbon atom from the methyl end and has the greatest EFA value for marine animals in general (Leaf *et al.* 2008; McVey 1993). Neither of these two families of fatty acids (FAs) is synthesized *de novo* by crustaceans and must be supplied in the diet (González-Félix & Perez-Velazquez 2002). Both LOA and LNA are precursors for the synthesis of a variety

of other unsaturated FAs. For instance, the n-6 highly unsaturated fatty acid (HUFA), arachidonic acid (AA; 20:4 (n-6)) which is derived from LOA is an essential precursor of eicosanoids including prostaglandin, thromboxanes and prostacyclins. These hormones are known to promote maturation and molting in penaeid shrimp (Davis 2005). The two n-3 HUFAs, eicosapentaenoic (EPA; 20:5 (n-3)) and docosahexaenoic (DHA; 22:6 (n-3)) acids are also important to penaeid shrimp and these two HUFAs have greater growth promoting effects in penaeid shrimp than LNA (Glencross & Smith 1999). In fishes, DHA is important in the functioning of the nervous system and provide immunity to infections and parasitic diseases (Kolakowska *et al.* 2002). A dietary n-3 HUFA input is needed to compensate the very limited capacity of bio-conversion of dietary LNA by marine fish and crustaceans (Wouters *et al.* 1997). Table 1.3 presents the recommended dietary EFAs in various penaeid species.

Table 1.3 Recommended dietary EFAs in various penaeid species (Perez-Velasquez *et al.* 2004).

Essential FAs	% on DW in feed		
	<i>P. japonicus</i>	<i>P. monodon</i>	<i>L. vannamei</i>
Linoleic acid (LOA; 18:2 (n-6))	0.25	1.5	0.1
Linolenic acid (LNA; 18:3 (n-3))	0.25	1.0-2.5	0.1
Eicosapentaenoic (EPA; 20:5 (n-3))	0.9 (combination of EPA and DHA)	0.9	0.4 (combination of EPA and DHA)
Docosahexaenoic (DHA; 22:6 (n-3))		0.9-1.44	
Total	1.5	4	0.8

Sterols are also important lipids that need to be provided in a diet of crustaceans as they are incapable of *de novo* sterol synthesis from acetate (Parrish 2013). Cholesterol is

the most important sterol in animals (Puskas 2013). In crustaceans, they are precursor for steroid hormones such as ecdysteroids that are primarily involved in molting and in regulating gonadal maturation particularly ovary maturation in crustaceans (Summavielle *et al.* 2003). The cholesterol levels in feed (% on DW) recommended for *P. indicus* range from 0.2-0.5 %, 0.17-1 % for *P. monodon*, 0.26-0.6 % for *P. japonicus* and 0.15 % in *L. vannamei* (Teshima 1997; Castille *et al.* 2004; Smith *et al.* 2001).

The growth-promoting effects of phospholipids are also demonstrated in penaeid shrimp (González-Félix & Perez-Velazquez 2002). Phospholipids are comprised of a group of polar lipids added to the diet of crustaceans as a source of energy and to emulsify lipids during digestion and absorption (Gong *et al.* 2001). Phospholipids are also vital to the normal functioning of every cell and organ. Its polar head group and two hydrophobic hydrocarbon tails make up the basic structure of a cell membrane (Alberts *et al.* 2002). The major phospholipids in animals are phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PSe). PC and PI are required while PE and PSe have no known nutritional effects in crustaceans (Takeuchi & Murakami 2007). Lecithin is the generic term presently used for various types of phospholipids. Although most crustaceans can synthesize phospholipids *de novo* (Shieh 1969), this biosynthesis generally cannot meet metabolic requirements during the juvenile stage and therefore, dietary phospholipids are required (Gong *et al.* 2001). In most shrimp diets, soybean is the most important commercial source of lecithin (Hertrampf 1991). The phospholipid levels in feeds (% on DW) recommended for most crustaceans ranges from 0.5-1 % (Takeuchi & Murakami 2007).

1.3.2 Protein and amino acids

Shrimp like any other animal, must consume protein to furnish a continuous supply of essential amino acids for growth and repair of tissues (Davis 2005). Proteins are large, complex molecules made up of amino acids that serve as the major organic materials in some animal tissues, constituting about 65-75 % on DW (Akiyama *et al.* 1991). In practice, shrimp feeds are formulated to contain high levels of protein making it one of the major and most expensive components of feeds (Kureshy & Davis 2000). Shiau (1998) provided a list of dietary protein requirements for growth of penaeid shrimp (Table 1.4).

Table 1.4 Protein requirements for various species of penaeid shrimp.

Penaeid species	Requirement (% on DW)
<i>P. aztecus</i>	40
<i>P. californiensis</i>	35
<i>P. indicus</i>	43
<i>P. japonicus</i>	50
<i>P. merguensis</i>	34–42
<i>P. monodon</i>	45–50
<i>P. setiferus</i>	28–32
<i>P. stylirostris</i>	35
<i>L. vannamei</i>	30

Although the optimal dietary protein levels can vary among shrimp species and protein sources (Wouters *et al.* 2001), it is generally recommended that shrimp feeds

should have a balance mixture of essential and non-essential amino acids (Akiyama *et al.* 1991). Ten amino acids are reported to be essential to penaeid shrimp species: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. These amino acids are also important to finfishes and terrestrial animals. Wouters *et al.* (2001) further recommend that dietary amino acid profiles found in maturation diets should be similar to those found in fresh foods such as squid, bloodworm and mussels.

1.4 Diseases as major constraint in penaeid shrimp culture

Many top shrimp producing countries have a large percentage of semi-intensive to intensive farming systems. The characteristics of these farming systems have been described in the earlier section (section 1.2). These systems basically use wide array of chemicals and biological products to enhance production and to solve problems associated with heavy nutrient loading, toxic metabolites, pathogens and to maintain optimal physico-chemical parameters required for aquatic animal growth (Shamsuzzaman & Biswas 2012).

Despite obtaining high levels of production, shrimp farmers have suffered from significant economic losses since two decades due to disease problems that plague the industry (Moss 1999). One example is the collapse of the Taiwan shrimp industry in the late 1980s (Feigon 2000). Taiwanese shrimp production reached 80 000 metric tons of *P. monodon* in 1987. In 1988, however, Taiwanese shrimp production was greatly reduced to 20 000 metric tons due to outbreaks of the monodon baculovirus (MBV). This resulted in an economic loss of US \$ 420 million (Stickney 2000). Thailand, the world's leading shrimp producer after Taiwan and China, also experienced the same fate when its

production dropped in 1996-1997. Outbreaks of white spot syndrome virus (WSSV) and yellowhead virus (YHV), as well as bacterial diseases resulted in the reduction of shrimp production from 240 000 metric tons in 1995 to 150 000 metric tons in 1997. Losses due to WSSV alone were estimated to be at US \$ 600 million (Chanratchakool & Phillips 2002). The same boom-bust pattern was also observed in the Philippines and in Indonesia (Primavera 1997). A survey on shrimp production by the Global Aquaculture Alliance (GAA) in 2001 revealed an estimated overall loss due to disease of 22 % in a single year. Given a total production of 700 000 metric tons in 2001 valued at approximately US \$ 8 per kg, this translates into an estimate of about US \$ 1 billion loss in a single year (Flegel 2006). This illustrates the importance of disease control to the industry.

Walker & Mohan (2009) identified several factors contributing to the emergence of diseases in the shrimp industry including the provision of artificial diets containing sub-optimal nutrients, stocking at too high density and exposure to stress imposed for example by sub-optimal water quality and transportation. Semi-intensive and especially intensive farming systems have high risk of disease outbreaks caused by viruses, bacteria, fungi or other pathogens (Gräslund & Bengtsson 2001). Figure 1.4 shows the relative economic loss related to disease caused by various pathogen groups. Common examples of these groups are provided in Table 1.5. Details on viral and bacterial pathogens are discussed in succeeding sections.

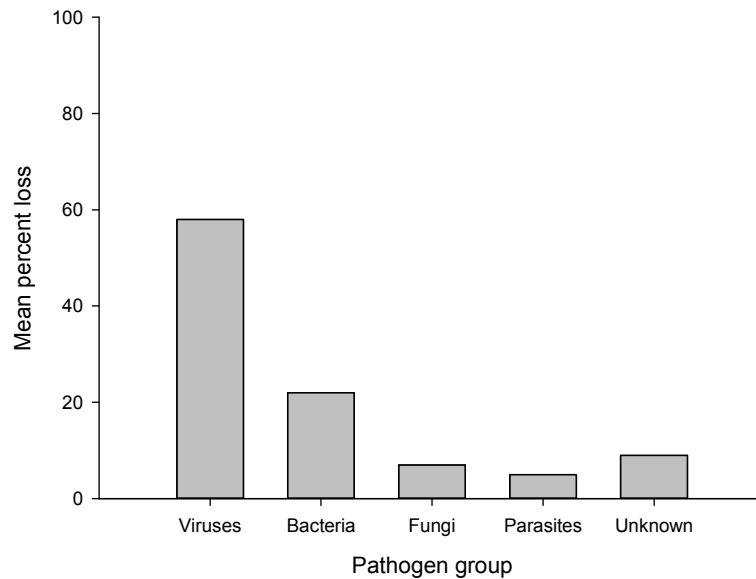


Figure 1.4 Relative economic loss in 2001 related to disease caused by various pathogen groups (Flegel *et al.* 2008).

Table 1.5 Common pathogens of penaeid shrimp (Lightner *et al.* 2009).

Groups	Common examples
Viruses	WSSV, YHV, baculoviral mid-gut gland necrosis virus (BMN), MBV, infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), reolike virus (REO), taura syndrome virus (TSV), baculovirus penaei (BP)
Bacteria	Vibriosis (septic hepatopancreas necrosis, hatchery vibriosis, syndrome gaviota, shell disease)
Fungi	Rickettsia, larval mycosis, fusariosis
Parasites	Epicommensal (<i>Leucothrix mucor</i> and peritrich protozoans), gregarines
Others	Microsporidians, nutritional imbalance, toxicants/contaminants, etc.

1.4.1 Viruses

In terms of economic impact, viral diseases stand out as the most significant infectious disease of cultured shrimp (Table 1.6 and 1.7).

Table 1.6 OIE (Office International des Epizooties)-listed penaeid shrimp diseases as of 2006 and those being considered for listing (Lightner 2011).

Disease name	Pathogen type	Pathogen name & acronym
Taura syndrome	ssRNA virus	TSV
White spot disease	dsDNA virus	WSSV
Yellowhead disease	ssRNA virus	YHV & gill-associated virus (GAV)
Tetrahedral baculovirus	dsDNA virus	BP
Spherical baculovirus	dsDNA virus	MBV
Infectious hypodermal and hematopoietic necrosis (IHHN)	ssDNA virus	IHHNV
Infectious myonecrosis (IMN) ^a	dsRNA virus	Infectious myonecrosis virus (IMNV)
Hepatopancreatic parvovirus disease ^b	ssDNA virus	HPV
Mourilyan virus disease ^b	ssRNA virus	Mourilyan virus (MOV)

^a Listing of this disease is under study by the OIE

^b Listed as emerging diseases by OIE

ssRNA = single-stranded ribonucleic acid

dsRNA = double-stranded ribonucleic acid

ssDNA = single-stranded deoxyribonucleic acid

dsDNA = double-stranded deoxyribonucleic acid

Table 1.7 Estimated economic losses since the emergence of viral diseases in penaeid shrimp (Lightner 2011).

Viruses	Year of emergence to 2001	Economic loss (in US \$) from emergence to 2001
WSSV (Asia)	1992	4.6 billion
WSSV (USA)	1999	> 1 billion
TSV	1991/1992	1-2 billion
YHV	1991	0.1-0.5 billion
*IHHNV	1981	0.1-0.5 billion

*Includes the Gulf of California fishery losses for 1989-1994.

Worldwide, there existed only 6 shrimp viruses in 1989 but this number rose to twenty in 1996 (Hernández-Rodríguez *et al.* 2001). The first large scale disease outbreak for MBV was recorded in Taiwan in 1988 (Flegel *et al.* 2008). This was followed by epidemics caused by IHHNV in the USA (Lightner 1996), YHV in Thailand (Flegel 1997) and TSV in the USA (Brock *et al.* 1997).

1.4.2 Bacteria

Bacterial infections in penaeid shrimp have been observed for many years. They occur at all shrimp life stages and are responsible for considerable economic losses in several countries (Vaseeharan *et al.* 2003). Most of the bacterial infections in shrimp aquaculture occur when shrimp are weakened or when the environmental conditions favor the presence and abundance of a particularly harmful bacterium (Saulnier *et al.* 2000). According to Abraham *et al.* (2003), bacterial infections in shrimp may take three general

forms: erosions of the cuticle covering the general body surface, gills, and appendages (bacterial necrosis and shell disease), localized lesions within the body and generalized septicemia. Lavilla-Pitogo (1995) identified *Leucothrix* sp. and several species of *Vibrio* as the two groups of bacteria having caused serious diseases in the various phases of the shrimp culture cycle in the Southeast Asian countries. The ectocommensal filamentous bacterium *Leucothrix* sp. has been found in high numbers attaching to the gill filaments in various penaeid species. In massive infestation, *Leucothrix* sp. interferes with gas diffusion across gill cuticle causing mortalities (Treece & Fox 1999). Other examples of filamentous bacteria causing infection to penaeid shrimp are *Thiothrix* sp., *Flexibacter* sp., *Flavobacterium* and *Cytophaga* sp. (Dinakaran *et al.* 2013). Vibriosis is another disease identified as a serious concern in shrimp aquaculture all over the world. *Vibrio* spp. are common members of the bacterial community in the marine environment (Strom *et al.* 2013) and constitute a substantial part of the normal microflora of farmed and wild penaeid shrimp (Costa *et al.* 1998). The outbreaks of a shrimp disease called “tea brown gill syndrome” (TBGS) caused by *V. harveyi*, a luminous bacteria, in association with a tailed bacteriophage was reported in Thailand in 1998 (Ruangpan *et al.* 1999). Recently, a new emerging disease in penaeid shrimp commonly referred as “early mortality syndrome” (EMS) or more technically known as “acute hepatopancreatic necrosis disease” (AHPND) was reported to affect shrimp farms in southern China in 2010 and subsequently in Vietnam, Thailand and Malaysia (FAO 2013b). The named EMS is coined due to mass mortality within 20-30 days after stocking (De Schryver *et al.* 2014). The causative agent of EMS/AHPND has been reported to be a bacterium—more specifically a

pathogenic *Vibrio* belonging to the Harveyi clade, presumably *Vibrio parahaemolyticus* (GAA 2013). Determination of the infectious nature of the agent of EMS is discussed in details elsewhere (Tran *et al.* 2013). The virulence of various species of *Vibrio* injected intramuscularly into healthy *P. monodon* juvenile is presented in Table 1.8.

Table 1.8 LC₅₀ values (48 h) of *Vibrio* spp. isolated from diseased shrimp (Jayasree *et al.* 2006).

Bacterial isolates	Diseases	LC ₅₀ concentration (CFU g ⁻¹ of tissue)
<i>V. harveyi</i>	Loose shell syndrome	1.0 x 10 ³
	Red disease	2.5 x 10 ⁴
	White gut syndrome	1.0 x 10 ⁴
<i>V. parahaemolyticus</i>	Red disease	1.5 x 10 ⁴
	Loose shell syndrome	4.0 x 10 ⁴
	Tail necrosis	3.0 x 10 ⁵
<i>V. alginolyticus</i>	Loose shell syndrome	4.5 x 10 ⁴
	Red disease	6.0 x 10 ⁴
	White gut syndrome	2.0 x 10 ⁴
	Shell disease	2.5 x 10 ⁵
<i>V. anguillarum</i>	Loose shell syndrome	5.0 x 10 ⁵
	Red disease	1.0 x 10 ⁶
	White gut disease	1.5 x 10 ⁶
	Shell disease	6.0 x 10 ⁶
	Tail necrosis	1.0 x 10 ⁹

Few studies have also documented the occurrence of black spot disease caused by a chitin-degrading bacteria *Beneckeia* sp. This disease is characterized by the breakdown of cuticular protection resulting in loss of haemolymph and invasion by other internally destructive pathogens (Couch 1978). Other gram-negative rods, including *Pseudomonas* spp. and *Aeromonas* spp. are also occasionally involved in bacterial disease syndromes in penaeid shrimp (Lightner 1983). Bacteria are able to invade hosts in numerous ways. For example, *Leucothrix* sp. attaches to the gill filaments by a mucus layer (Meurant 1990) while *Vibrio* spp. often infect body fluids of penaeid shrimp. Some group of bacteria can also enter orally and invade through the digestive tract (Soonthornchai *et al.* 2010). They can also penetrate through the gills, through insertion of the setae or through damaged cuticle (Alday-Sanz *et al.* 2002).

1.5 Shrimp disease management strategies

Most of the management efforts in penaeid shrimp industry mainly focus in protecting the organism from becoming infected, carrying and spreading diseases (Walker & Mohan 2009). In general terms, the resources for disease control applicable in penaeid shrimp culture should include quarantine and improvement of biosecurity that will reduce the probability of pathogen introduction and subsequent spread from one place to another (Pruder 2004).

1.5.1 SPF and SPR

The development of specific pathogen-free (SPF) stocks is probably a viable long term solution to exclude specific pathogens in penaeid aquaculture. SPF shrimp are

selected through a multigenerational process that includes strict quarantine, constant sampling and histopathological analysis for the absence of the pathogens of concern (Newman 2009). According to Lotz (1997), the process of obtaining SPF shrimp involves the capture of apparently healthy wild stock from areas of low disease prevalence followed by primary quarantine in which individuals are screened for specific pathogens. The contaminated individuals are destroyed. Next, SPF shrimp is transferred to secondary quarantine where it is reared to broodstock size and monitored regularly. Finally, disease-free broodstock is transferred to breeding centers for production of multiple families from different sources. Larvae produced from SPF broodstock are reared in bio-secure hatcheries from the selected families and any infected stock detected through continuous monitoring are immediately discarded. Currently, lines of SPF *L. vannamei* are commercially available in the USA, but these are only free from TSV and are not necessarily free from other viruses. A number of programs in Hawaii, Thailand and Australia are also developed to produce SPF *P. monodon* broodstock. Although significant success has been achieved, commercial availability of stocks is limited (Barman *et al.* 2012). Another drawback with SPF stocks is that it provides no guarantee against the animal being infected with unknown pathogens or known pathogens within the system (FAO 2005).

Specific pathogen-resistant (SPR) describes a genetic trait of a shrimp that confers some resistance against one specific pathogen and usually result from a specific breeding programme designed to increase resistance to a particular virus (FAO 2005). According to GAA (2003), SPR animals are not susceptible to infection and present no risk of

transmission of the particular pathogen, provided there is no “mechanical” contamination through any viable pathogen adhering to the gills or other surfaces. Some works on the development of SPR shrimp include the selective breeding programme for *L. vannamei* that was initiated in 1995 in the Oceanic Institute in Hawaii based on a selection index weighted equally for growth and resistance to Taura Syndrome Virus (TSV). Shrimp selected for TSV resistance exhibited a mean family survival that was 18.4 % higher than unselected control shrimp after a TSV-challenge test (Argue *et al.* 2002). WSSV-resistant *P. chinensis* has been introduced by Jie *et al.* (2003). These authors found improved survival survival rate from 0–0.8 % to 12–45 % from ponds stocked with PL produced from survivors of a WSSV epidemic. In general, selective breeding for resistance is an attractive option for managing diseases, but the development of genetically disease resistant populations entails high cost and therefore, this approach is only advisable when: (a) the disease causes severe damage; (b) there are no other existing simple cost effective control measures; and (c) there is demonstrable genetic variation in resistance and this is not coupled with an excessive level of negative associations with other desirable characteristics (Cock *et al.* 2009).

1.5.2 Chemicals

Chemicals in penaeid shrimp aquaculture have been routinely applied as prophylactics to prevent diseases and/or as therapeutants to control diseases once detected. Based on their actions, chemotherapeutic agents used in aquaculture system can be grouped into: antibacterial agents including antibiotics and disinfectants, anti-protozoal

agents and metazoan parasitocides (Liao 2000). Antibiotics and disinfectants are discussed in the succeeding texts.

In Asia, antibiotics and disinfectants are commonly used in larval and growth phases (Ma *et al.* 2006). Antibiotics which are derived from bacteria, molds and *Actinomyces*, selectively inhibit or destroy pathogenic organisms without showing substantial effects to the host while disinfectants are chemical products that are commonly applied in biosecurity programmes designed to exclude specific diseases, as well as a routine sanitary measure employed to reduce disease incidence within farms including disease eradication (Shamsuzzaman & Biswas 2012).

Examples of antibiotics commonly used in aquaculture to combat bacterial diseases, include oxytetracycline, florfenicol, sarafloxacin and enrofloxacin (Roque *et al.* 2001; Soto-Rodríguez *et al.* 2006). Other antibiotics such as chlortetracycline, quinolones, ciprofloxacin, norfloxacin, oxolinic acid, perfloxacin, sulfamethazine, gentamicin, and tiamulin are also used globally (Holmström *et al.* 2003). Common examples for disinfectants are iodine (e.g., povidone-iodine, iodophor) and chlorine compounds (e.g., sodium hypochlorite, chlorinated lime) that are frequently applied in disinfecting and washing fertilized eggs or to dip aquatic animals when they are transferred from one aquarium or pond to another. These compounds are also used to disinfect tanks and other holding equipment (Liao 2000). In general, the application of antibacterial agents particularly antibiotics, is prohibited due to its potential negative impacts in the environment (Biao & Kaijin 2007) and human health, including bacterial resistance, persistence of the disease in the aquatic environment and effects on the biogeochemical

composition of the sediment (Bermúdez-Almada & Espinosa-Plascencia 2012). The accumulation of antibiotic residues in the edible tissues of shrimp may also alter human intestinal flora and cause food poisoning or allergy problems (Ma *et al.* 2006). The application of disinfectant primarily chlorine compounds is thought to result in the potential formation of toxic by-products such as halogenated hydrocarbon that are environmental contaminants and, at times, can bioaccumulate in the food chain (Gräslund *et al.* 1999).

1.5.3 Probiotics

Interest on the application of probiotics to control diseases in aquaculture is increasing (Wang *et al.* 2005). Probiotics, as defined by Verschuere *et al.* (2000), are live beneficial microbial actors whose application originally exclusively aimed at improving the intestinal balance to increase the health status of the aquatic animals. They may also compete for nutrients necessary for the survival of pathogens or prevent their adhesion to the host's intestine. In 1998, Moriarty reported that the use of probiotic strains of *Bacillus* spp. at a density of 10^4 - 10^5 cells mL⁻¹ increased the quality and viability of giant tiger shrimp cultured in ponds in Jakarta (Indonesia). Wang *et al.* (2005) used commercially available probiotics containing a total bacterial density of 10^9 CFU mL⁻¹ with *Bacillus* sp. and photosynthetic bacteria as the two most dominant bacteria. Based on their results, bacterial density of presumptive vibrios in *L. vannamei* ponds without the addition of probiotics was 2.1×10^3 cells mL⁻¹ while this was 4.4×10^2 cells mL⁻¹ in ponds added with probiotics. During harvest, ponds receiving no probiotics had a total production, feed conversion ratio (FCR) and survival of 4985 kg ha⁻¹, 1.35 and 48 %, respectively.

respectively, while these were 8215 kg ha⁻¹, 1.13 and 82 % in ponds receiving the probiotics. In general, probiotics have been shown to have positive effects, however, inactivation during the manufacturing process is one of its major drawbacks. To overcome this, Qi *et al.* (2009) suggested to improve the technology involved in screening resistant strains or alternatively, by protecting the probiont through micro-bioencapsulation. It was also suggested by Gomez-Gil *et al.* (2000) that selection of strains needs to be adapted for different host species and environments.

1.5.4 Prebiotics

Prebiotics are defined as non-digestible food ingredients that selectively stimulate the growth and/or the metabolism of health-promoting bacteria in the intestinal tract, thus improving an organism's intestinal balance (Gibson & Roberfroid 1995). According to Mahious & Ollevier (2005), any foodstuff that reaches the colon, e.g. non-digestible carbohydrates, some peptides and proteins, as well as certain lipids, is a candidate prebiotic. Inulin and oligofructose are among the most known and well-studied prebiotics in human and terrestrial animals (Mahious *et al.* 2006). Hoseinifar *et al.* (2015) investigated the effects of inulin as prebiotic on gut microbiota of Indian white shrimp *Fenneropenaeus indicus*. Significant increase in the survival rate and levels of lactic acid bacteria (LAB) in the postlarval gut were seen. According to the authors, the latter is attributed to inulin as substrate for growth of LAB. The use of commercial prebiotic GroBiotic®-A, which is a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, was tested by Li *et al.* (2009) and this was

found to improve the survival of whiteleg shrimp *L. vannamei*. However, Hai & Fotidar (2009) did not find significant improvement in specific growth rate (SGR), FCR and survival of Western king shrimp *P. latisulcatus* juveniles fed diet supplemented 0.5 % Bio-Mos consisting of mannan-oligosaccharide derived from yeast cell wall and 0.2 % β -1,3-D-glucan. The reasons for the different results are not clear but differences in basal diet, inclusion level, origin of prebiotic and animal characteristics such as species and developmental stages may have contributed to this. Therefore, comprehensive research to fully characterize the intestinal microbiota of target species and their responses to prebiotics are suggested to render prebiotics more effective (Yousefian *et al.* 2009).

1.5.5 Immunostimulant

Immunostimulants are naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell & Dalmo 2005). According to Apines-Amar & Amar (2015), immunostimulants can be obtained from several sources including bacterial derivatives (e.g. lipopolysaccharide (LPS)), complex carbohydrates (e.g. glucans), nutritional factors (e.g. carotenoids and trace elements such as zinc, copper, manganese and selenium), animal extracts (e.g. chitin/chitosan), plant extracts and hormones/cytokines (e.g. lactoferrin). In vertebrates, synthetic immunostimulant such as levamisole that was originally produced as an anthelmintic, appears to restore depressed immune function of B lymphocytes, T lymphocytes, monocytes and macrophages (Shah *et al.* 2011). Examples of commercially available immunostimulants are presented in Table 1.9.

Table 1.9 Commercially available immunostimulants (Apines-Amar & Amar 2015).

Product	Company	Active components
AQUAVAC® Ergosan™	Merck Animal Health	Algins and polysaccharide
BZT® PRE-GE	United Tech	Mannan oligosaccharide and β -glucan
Vannagen®	Chemoforma Ltd.	Nucleotides
Bio-Mos®	Alltech	Outer cell wall of <i>Saccharomyces cerevisiae</i> yeast
Immustim®	Immudyne, Inc.	β -1,6 branched and β -1,3-glucan from yeast
MacroGard®	Biotce-Mackzymal, Norway	β -1,6 branched and β -1,3-glucan from yeast
Levamisole	Janssen Pharmaceutica, Belgium	Tetrahydro-6-phenylimidazolthiazole Hydrochloride
VitaStim	Taito Co., Japan	β -1,6 branched and β -1,3-glucan from fungi
EcoActiva™	Ecostadt	β -1,6 branched and β -1,3-glucan from yeast
Sanoguard® S-PAK	INVE Aquaculture Health	Combination of vitamins, nucleotides and various immunostimulants

The documented actions of immunostimulants in shrimp include upregulation of immune indices such as total hemocyte count, respiratory burst, phenoloxidase (PO) activity, phagocytic activity, agglutination titer, lysozyme and superoxide dismutase (SOD) activities (Lee & Shiau 2002; Sivakumar & Felix 2011; Lee & Shiau 2004; Lin *et al.* 2013). The effectiveness of β -1,3-glucan derived from the fungus *Schizophyllum*

commune on pond-reared giant tiger shrimp adults (135 ± 25 g) maintained either in indoor or outdoor tank was evaluated by Chang *et al.* (2000). Regardless of indoor or outdoor rearing, adults fed a test diet containing β -1,3-glucan at 2.0 g kg^{-1} showed enhanced haemocyte phagocytic activity, cell adhesion and superoxide anion production as compared to adults fed glucan-free control diet for 40 days. Results in whiteleg shrimp (13 ± 1 g) fed a diet containing 0.05 % mushroom beta-glucan (M β G) extracted from *Ganoderma lucidum* and *Coriolus versicolor* showed significant increase in total haemocyte count (THC) and semi-granular cells ratio at 28 days as compared to shrimp fed a diet containing no MBG (Yang *et al.* 2014).

As with probiotics, most of the commercially available immunostimulants have been tested and shown to be effective. However, its effects are normally short-lived (Sakai 2015). In addition, the effects of immunostimulants are not directly dose dependent, and high dose or overdosage may not enhance and may even inhibit immune responses (Galeotti 1998). Therefore, additional research is needed to define the specific dosage rates and efficacy of various immunostimulants for a variety of aquatic species. Strategies to reduce its costs must also be developed as many of these immunostimulants are expensive, limiting the ability to rapidly screen the potential immunostimulant regimens for efficacy (Barman *et al.* 2012).

1.5.6 Shrimp vaccines

In general, vaccines are special case of immunostimulation in that it contain a specific antigen and thus trigger the specific immune system to protect animals against

infections at a later stage (Sakai 1999). Shrimp, just like the other invertebrates, lack true adaptive immunity and it solely depends on innate immunity so that the development of vaccine against shrimp pathogens is hindered by the lack of memory type immunity (Musthaq & Kwang 2011). However, Flegel & Pasharawipas (1998) suggested an active viral accommodation by the shrimp immune system that allows the virus to replicate without causing any damage to or response from the host. In 2000, Venegas and co-workers demonstrated the existence of acquired resistance (quasi-immune response) in *P. japonicus* infected naturally or experimentally with WSSV. In their study, the survivors of natural WSSV outbreak were re-challenged after 4 months and demonstrated 94 % relative percent survival (RPS). In 2004, Namikoshi *et al.* investigated the efficacy of formalin-inactivated WSSV and of the two recombinant proteins of WSSV (rVP26, rVP28) by intramuscular injection followed by intramuscular challenge of *P. japonicus* with WSSV. Shrimp injected with formalin-inactivated WSSV showed resistance to the virus at day 10 post-injection with RPS of 50 %. However, this decreased to 5 % on day 30. Injection with rVP26 and rVP28 induced higher resistance, with RPS values of 60 % and 95 %, respectively, in *P. japonicus* at day 30 post-injection.

Although promising results have been obtained on the application of shrimp vaccines, improving its protection rate as well as sustaining its efficacy for extended duration after exposure to pathogen remained a challenge. One strategy that might be possible to overcome this hurdle is by expressing envelope protein in eukaryotic expression system. This could also act as a delivery vehicle or vector vaccine (Musthaq &

Kwang 2011). Finally, development of practical and cost-effective methods for application for commercial scale should also be taken into consideration.

1.5.7 Phage therapy

The application of bacterial viruses (bacteriophages) in deactivating pathogenic bacteria is also investigated in penaeid shrimp. Vinod *et al.* (2006) examined the lytic activity of a bacteriophage against *V. harveyi*. This bacteriophage belonging to the family Siphoviridae was isolated from shrimp farm water in the west coast of India. Addition of this bacteriophage at 100 µl containing 10^9 plaque-forming units (PFU) mL⁻¹ at 0 and 24 h significantly increased the survival of postlarval tiger shrimp from 25 % for *V. harveyi*-infected postlarvae without the provision of bacteriophage to 80 %. In 2007, Karunasagar *et al.* examined the lytic capacity of three bacteriophages isolated from oyster tissue and one from shrimp hatchery water against *V. harveyi*. All bacteriophages lysed 55–70 % of the 100 *V. harveyi* isolates tested.

Despite the potential of using bacteriophages as antibacterial agents in aquatic environment, concerns have been raised on its application for a number of reasons including the conversion of phage-sensitive bacteria into insensitive ones and therefore, proper selection and determination of effective doses should be taken into consideration (Loc-Carillo & Abedon 2011).

1.6 Poly- β -hydroxybutyrate (PHB): A new strategy for sustainable aquaculture

Penaeid shrimp aquaculture, just like any other industry, constantly requires new technologies in order to increase production yield. Recently, the use of the bacterial storage compound poly- β -hydroxybutyrate (PHB) as a new strategy in aquaculture showed promising results in terms of improving survival and overall performance of the tested aquatic farmed species (Defoirdt *et al.* 2007b; Sui *et al.* 2012; Suguna *et al.* 2014; Halet *et al.* 2007; Laranja *et al.* 2014; Thai *et al.* 2014; Nhan *et al.* 2010; De Schryver *et al.* 2010a).

1.6.1 The family of polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates that are synthesized by many gram-positive and gram-negative bacteria from at least 75 different genera. These polymers are accumulated intracellularly to levels as high as 90 % of the cell DW mainly under conditions of nutrient stress and act as a carbon and energy reserve (Madison & Huisman 1999). Common examples of pure and industrial bacterial strains commonly used for pilot and large scale production of PHAs are presented by Chen (2009). According to Khanna & Srivastava (2005), bacteria capable of synthesizing PHA can be divided into two groups: bacteria requiring limitation of an essential nutrient such as nitrogen, phosphorus, magnesium or sulphur combined with an excess in carbon source and bacteria which do not require nutrient limitation for PHA to accumulate during growth and stored as intracellular product. The reason for PHA accumulating in the second group is the low activity of the β -ketothiolase, which catalyzes the cleavage of acetoacetyl-CoA

(Vasylchenko *et al.* 2011). Notable examples of the former group are *Alcaligenes eutrophus*, *Protomonas extorquens* and *Protomonas oleovorans* while *A. latus* and the genetically modified *Azotobacter vinelandii* mutant strain and recombinant *E. coli* containing PHA biosynthetic genes are representatives for the latter.

The fermentation strategies employed for fed batch culture of bacteria belonging to the first group involved a two step cultivation method: cells are grown to desired concentration without nutrient limitation, after which an essential nutrient is limited to allow PHA synthesis. In this method, PHAs are primarily produced from carbon assimilation (from glucose or starch) and are used by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available (Khanna & Srivastava 2005). Examples of fermentation strategies for the production of PHAs in bacteria requiring limitation of essential nutrient are presented by Amache *et al.* (2013). The fed batch culture of bacteria belonging to the second group requires development of efficient nutrient feeding strategy. Here, a complex nitrogen source such as yeast extract and fish peptone can be supplemented to the cells to enhance cell growth as well as PHA accumulation. Cell growth and PHA accumulation need to be balanced to avoid incomplete accumulation of PHA or premature termination of fermentation at low cell concentration (Alavi *et al.* 2014). The main advantage of growth-associated PHA accumulation is the short fermentation time and avoidance of extra operations associated with the two-step fermentation process for PHA-accumulation under nutrient limitation (Vasylchenko *et al.* 2011).

1.6.2 Synthesis of PHB

PHB, a linear polyester of D(-)-3-hydroxybutyric acid, was the first PHA discovered in 1920s by the French bacteriologist Maurice Lemoigne (Lenz & Marchessault 2005). Its biosynthesis in bacteria requiring nutrient limitation involves three enzymatic reactions catalysed by three different enzymes (Zinn *et al.* 2001). The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by the enzyme β -ketothiolase encoded by *phbA* genes. This reaction is followed by the reduction of acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA by an NADPH (nicotinamide adenine dinucleotide phosphate)-dependent acetoacetyl-CoA reductase encoded by *phbB* gene. Finally, the (R)-3-hydroxybutyryl-CoA is polymerized into poly- β -hydroxybutyrate by PHB synthase encoded by *phbC* gene. This cycle becomes activated when acetyl-CoA is restricted from entering the tricarboxylic acid (TCA) cycle due to nutrient limitation such as nitrogen, phosphorous or oxygen needed by the cell to further metabolize acetyl-CoA for cell growth (Lenz & Marchessault 2005). The synthesis of PHB by recombinant *E. coli* does not require limitation of specific nutrient, but is dependent on the amount of acetyl-CoA available (Khanna & Srivastava 2005).

1.6.3 Properties of PHB

PHB contains repeating units of (R)-3HB (Sudesh *et al.* 2000). The monomers are polymerized into high molecular weight polymers in the range of 200 000 to 3 000 000 daltons (Da), depending on the microorganism and growth conditions (Byrom 1994). Kusaka *et al.* (1997) also discovered a PHB with a molecular weight of up to

20 megadaltons (MDa) produced out of a recombinant strain of *E. coli*. Inside the cell, PHB exists in a fluid, amorphous state called native PHB (nPHB) which is about 0.2-0.5 μm in diameter (Figure 1.5) (Porter & Yu 2011) and is covered by a surface layer consisting of proteins (so-called phasins) and phospholipids as shown in Figure 1.6 (Handrick *et al.* 2004). The major fraction of the proteins of the granule surface layer consists of relatively small amphiphilic polypeptides that presumably have a structural function between the water-insoluble polymer core and the hydrophilic cytoplasm. Native PHB inclusions (and PHAs in general) can be stained with Sudan black B indicating their lipid nature (Sudesh *et al.* 2000). Native PHB, once released from the cell (e.g. after cell lysis or after recovery by solvent extraction), denatures and becomes partially crystalline (55-80 %) (Merrick *et al.* 1965). The extracellular, partially crystalline PHB without a surface layer or with a damaged surface layer is called denatured PHB (dPHB) (Gebauer & Jendrossek 2006). X-ray studies on PHB crystal structure have shown that PHB forms lath-shaped crystals (slender and elongated form) with a dimension of 0.3–2 μm for the short and 5–10 μm for the long axes (Sudesh *et al.* 2000). The crystallization of PHB after extraction from bacterial cells was thought to result from physical treatments such as centrifugation. During centrifugation, PHB inclusions readily coalesce into larger masses accelerating crystallization (Sanders 1993). Furthermore, damages to the surface coating of the PHB inclusion allow heterogeneous nucleation, i.e. crystallization induced by external molecules other than PHA, further accelerating crystallization (De Koning & Lemstra 1992). Other physical treatments such as melting or solvent casting usually result in highly

crystallized PHB because of its stereoregular nature (Mark 2013). An overview on PHB extraction methods is described by Tan *et al.* (2014).

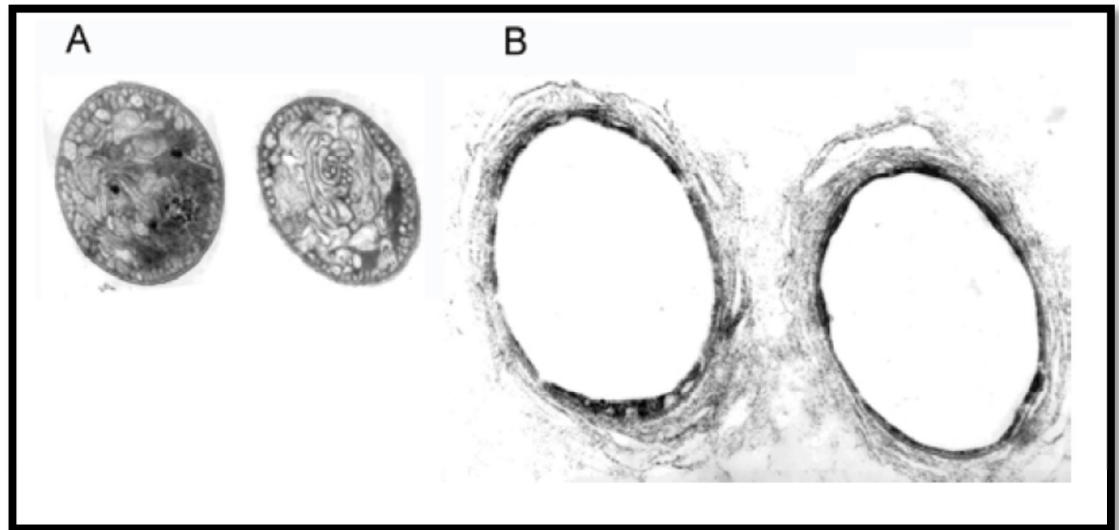


Figure 1.5 Transmission electron microscopy observation of *Azotobacter chroococcum*. 0 % PHB content (A) and 85 % PHB content (B) (Bonartsev *et al.* 2007).

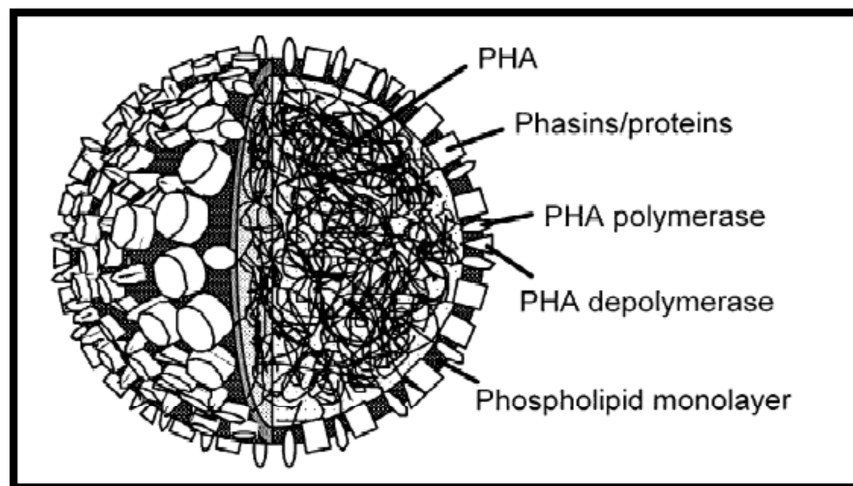


Figure 1.6 Scheme of a PHB granule (Zinn *et al.* 2001).

1.6.4 Degradation of PHB

1.6.4.1 Microbial degradation

One of the valuable properties of PHB is its biodegradability and any research on its biodegradation should clearly distinguish between intracellular PHB degradation and extracellular PHB degradation. Intracellular degradation is the active degradation (mobilization) of an endogenous PHB storage reservoir by the accumulating bacterium itself. Conversely, extracellular degradation is the utilization of exogenous PHB by a not-necessarily accumulating microorganism that secretes extracellular PHB depolymerase. Here, the source of extracellular polymer is PHB released by accumulating cells after death and cell lysis (Sudesh *et al.* 2000). Most enzymes that hydrolyze PHB are specific for one of the two forms of PHB. Intracellular PHB depolymerases are specific for nPHB and do not hydrolyze dPHB while extracellular PHB depolymerases are released from PHB-degrading bacteria that hydrolyze dPHB as an exogenous carbon source (Jendrossek & Handrick 2002).

The intracellular biodegradation pathway of PHB involves the depolymerization of nPHB by nPHB depolymerase. This step produce *R*-3-hydroxybutyric acid or *R*-3HB oligomers. *R*-3HB is further depolymerized by oligomer hydrolase (dimer hydrolase) to *R*-3HB monomer. *R*-3HB is dehydrogenated with NAD^+ (nicotinamide adenine dinucleotide) into acetoacetic acid which follows esterification with CoA-SH (Coenzyme A) to produce acetoacetyl-CoA by the action of acetoacetyl-CoA synthase with the aid of adenosine triphosphate (ATP). The acetoacetyl-CoA is degraded into acetyl-CoA by β -ketothiolase

and this compound enters the TCA and transformed to carbon dioxide and water under aerobic conditions (Smith 2005).

The extracellular biodegradation of PHB is important in the environment. Since PHB (or PHA in general) is a solid polymer with a high molecular weight and is incapable of being transported through the cell wall, various microorganisms excrete extracellular PHB depolymerases (dPHB depolymerase) that hydrolyse PHB into water-soluble oligomers and monomers and subsequently utilise these resulting products as nutrients within the cells. The biochemical and molecular characterisation of dPHB depolymerases have shown that all enzymes are comprised of N-terminal catalytic domain, a C-terminal substrate binding domain which is hydrophobic and a linker region connecting the two domains (Jendrossek *et al.* 1995). The substrate-binding domain of the dPHB depolymerase adsorb onto the water-insoluble polymer chain mainly by hydrophobic interactions while the catalytic domain is responsible for the hydrolyzing of ester bonds of dPHB (Smith 2005). The linker regions connecting the catalytic and substrate-binding domains have been identified in all known dPHB depolymerases and play a structural role in maintaining an optimal distance between the catalytic domain and substrate-binding domain. The enzymatic hydrolysis of dPHB by the dPHB depolymerase produces oligomers, mainly the dimers, and a small amount of β -HB monomer (Sudesh *et al.* 2000). Examples of microorganisms capable of degrading dPHB are presented in Table 1.10.

Table 1.10 PHB (or PHA in general) degrading microorganisms isolated from various environments (Khanna & Srivastava 2005).

Sources	Microorganisms
Soil	<i>Aspergillus fumigatus</i>
	<i>Acidovorax faecalis</i>
	<i>Comamonas</i> sp.
	<i>Paucimonas lemoignei</i>
	<i>Variovorax paradoxus</i>
Activated sludge	<i>Alcaligenes faecalis</i>
	<i>Pseudomonas fluorescens</i>
Sea water	<i>Comamonas testosteroni</i>
Lake water	<i>Pseudomonas stutzeri</i>
Anaerobic sludge	<i>Ilyobacter delafieldii</i>

1.6.4.2 Thermal degradation

PHB thermally decomposes at temperatures just above its melting point (180 °C), making processing difficult and limiting its commercial usefulness. A short exposure of PHB to 180 °C could induce severe degradation accompanied by production of olefinic and carboxylic acid compounds resulting in a drastic reduction in molecular weight (Rudnik 2010). Thermal degradation of PHB at 280 °C produces volatile products such as crotonic acid. Dimers and trimers are also found (Kopinke *et al.* 1996). When heated to 338 °C under vacuum, PHB releases isocrotonic acid (0.9 wt %), crotonic acid (35.3 wt %)

and the dimer (41.2 wt %), trimer (12.5 wt %) and tetramer (2.9 wt %) of PHB. When heating is continued to 500 °C, traces (4 wt %) of the degradation products of these volatiles are observed: carbon dioxide, propene, ketene, acetaldehyde and β -butyrolactone (Rudnik 2010). The thermal degradation of PHB has been suggested to occur almost exclusively via a random chain scission (cis-elimination) of the ester group, which has a six-member ring ester intermediate (Bugnicourt *et al.* 2014). To control thermal degradation during processing, suitable additives are used (Kaplan 2013). The production of PHB blends to reduce thermal degradation is discussed in the succeeding texts.

1.6.4.3 Non-enzymatic degradation

The non-enzymatic hydrolysis is important in the use of PHB as implant materials (Choi *et al.* 2004) and in controlled drug release systems (Chen & Wu 2005). In animal tissues, the rates of PHB degradation are influenced by the characteristics of the polymer such as chemical composition, structure and molecular weight (Abe & Doi 2002). In general, it has been suggested that the non-enzymatic hydrolysis of PHB proceeds in two stages. Initially, the chain scission takes place in the amorphous regions of the polymer resulting in the increase of crystallinity. Finally, crystallinity decreases as hydrolysis leads to degradation of chains in the crystalline regions (Gogolewski *et al.* 1993). In aqueous media, the non-enzymatic hydrolysis of PHB proceeds via a random bulk hydrolysis of the ester bond in the polymer chain films and occurs throughout the whole film, since water permeates the polymer matrix (Koyama & Doi 1995).

1.6.5 The practical applications of PHB

1.6.5.1 PHB as biodegradable plastic

Concerns about the non-degradable and non-renewable nature of petrochemical plastics have led to the search for degradable alternatives that can be produced at an industrial scale for common use (Thompson *et al.* 2009). PHB and other forms of PHA are suitable replacements for the petrochemically produced bulk plastics (polyethylene terephthalate (PET), polypropylene (PP), etc.) (Bonartsev *et al.* 2007). In contrast to the oil-based plastics, PHB is completely degraded into carbon dioxide and water through natural microbiological mineralization (Braunegg *et al.* 1998). It also possesses better physical properties than PP for food packaging applications and most importantly, it is completely non-toxic (Ansari & Fatma 2014). In addition, the high crystallinity (50-70 %) and excellent gas barrier nature of PHB make it suitable for industrial applications (Pachekoski *et al.* 2009). PHB is also water insoluble and relatively resistant to hydrolytic degradation. This differentiates PHB from most other currently available bio-based plastics which are either moisture or water soluble (Ebnesajjad 2012).

Despite the advantages of PHB, several drawbacks have been reported. The high stereoregularity of PHB makes it a highly crystalline polymer that is stiff and brittle. It is also thermally unstable during processing and its extension to break is markedly lower than that of PP. This unfortunate aspect of properties poses a limitation to, for example, the application to a flexible film, which is one of the largest uses of biodegradable polymers (Rudnik 2010).

One of the main approaches to improve the properties of PHB is the production of derivatives based on PHB via the biosynthesis of copolyesters containing PHB units with other 3-hydroxyalkanoates units (Bugnicourt *et al.* 2014). For example, the copolymer poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (P(3HB-co-3HV)) has lower crystallinity and improved mechanical properties (decreased stiffness and brittleness, increased tensile strength and toughness) compared to PHB, while still being readily biodegradable. This copolymer has also higher melt viscosity, which is a desirable property for processing (i.e. extrusion blowing) (Rudnik 2010). The blend of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHx) has improved mechanical properties and a lower melting point which prevent degradation during processing (Qui *et al.* 2005). The addition of plasticizers is also considered as a relatively simple route to modify the thermal and mechanical properties of PHB (El-Hadi *et al.* 2002). According to Cadogan & Howick (1996), plasticizers are materials which, when added to a polymer, cause an increase in the flexibility and workability, brought about by a decrease in the glass transition temperature (T_g) of the polymer. Examples of commonly used plasticizers are oxypropylated glycerin (or laprol), glycerol, glycerol triacetate, 4-nonylphenol, 4,40-dihydroxydiphenylmethane, acetyl tributyl citrate, salicylic ester, acetylsalicylic acid ester, soybean oil, epoxidized soybean oil, dibutyl phthalate, triethyl citrate, dioctyl phthalate, dioctyl sebacate, acetyl tributyl citrate, di-2-ethylhexylphthalate, tri(ethylene glycol)-bis(2-ethylhexanoate), triacetine, and fatty alcohols with or without glycerol fatty esters, polyethylene glycol (PEG) (Bugnicourt *et al.* 2014). Another approach to improve the

properties of PHB is to mix it with another polymer, not necessarily a biodegradable one such as that employed by Pachekoski *et al.* (2009) and Wellen *et al.* (2015).

1.6.5.2 PHB as medical implant materials

PHB is a promising material for tissue engineering due to its high biocompatibility *in vivo* (Bonartsev *et al.* 2007). PHB and its blends (e.g. poly-beta-hydroxybutyrate–hydroxyvalerate (PHBV), poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P3HB4HB), and poly(3-hydroxyoctanoate) (P3HO)) are also commonly investigated as use for repair devices, repair patches, slings, cardiovascular patches, orthopedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone marrow scaffolds, artificial esophagus and wound dressing (Chen & Wu 2005). Recently, PHBHHx was successfully used as osteosynthetic material for stimulating bone growth owing to its piezoelectric properties, as well as for repairing damaged nerves (Wang *et al.* 2008). The *in vivo* biodegradation of PHB implants is listed by Vasile & Zaikov (2009).

1.6.5.3 PHB as drug delivery carrier

The first drug-sustained release system using PHB was investigated by Korsatko *et al.* (1983) who observed rapid release of encapsulated drug (7-hydroxyethyltheophylline) from tablets of PHB with a molecular mass of 2000 kilodaltons (kDa). They also observed weight loss of PHB tablets containing the drug after subcutaneous implantation. Bonartsev *et al.* (2007) designed a novel system for sustained delivery of the anti-proliferative (dipyridamole (DP)) and anti-inflammatory (indomethacin) drugs using PHB films. It was

suggested that the release of the drugs occurs via two mechanisms: diffusion and degradation which operate simultaneously. According to these authors, the coefficient of release of the drugs was influenced by the thickness of the PHB films containing the drug, the weight ratio of dipyridamole and indomethacin in the polymer and the molecular weight of PHB. The PHB homopolymer and copolymer mainly P(3HB-co-3HV) and P3HB4HB have also been used for sustained drug delivery of antibiotics (rifampicin, metronidazole, ciprofloxacin, levofloxacin), anti-inflammatory drugs (flurbiprofen, dexamethasone, prednisolone), and antitumor drugs (paclitaxel).

1.6.6 Application of PHB in aquaculture

The application of PHB in aquaculture was first demonstrated by Defoirdt *et al.* (2007b) based on literature reports mentioning that PHB can be degraded into β -hydroxy short chain FAs (SCFAs). In their experiment on axenic *Artemia* nauplii, the provision of PHB particles to brine shrimp *Artemia franciscana* nauplii offered preventive protection against the pathogenic *V. harveyi* BB120 strain. It did not only offer protection against the pathogen, PHB also enhanced the survival of starved nauplii indicating that PHB could also be used as an energy source. These results provided the first indication on the potential application of PHB as a new strategy in aquaculture. An overview of studies conducted to explore the potential beneficial effects of PHB in various aquatic farmed species is presented in Table 1.11.

Table 1.11 Overview of published studies on the application of PHB in various aquatic farmed species.

Species name	Developmental stages	PHB form	Effects on host and microbial community	Reference
<i>Artemia franciscana</i>	Newly-hatched	Crystalline PHB	Host <ul style="list-style-type: none"> Enhanced the survival in starved and <i>Vibrio</i>-challenged <i>Artemia</i> nauplii Microbial community: N.R.	Defoirdt <i>et al.</i> (2007b)
<i>A. franciscana</i>	Newly-hatched	PHB-accumulating enrichment culture	Host <ul style="list-style-type: none"> Enhanced the survival of <i>Vibrio</i>-challenged <i>Artemia</i> nauplii Microbial community: N.R.	Halet <i>et al.</i> (2007)
European sea bass (<i>Dicentrarchus labrax</i>)	Juveniles	Crystalline PHB supplemented at varying levels in the diet	Host <ul style="list-style-type: none"> Enhanced the survival at 2, 5 and 10 % PHB supplementation Highest weight gain and lowest FCR at 5 % PHB supplementation Decreased the intestinal pH at any levels of PHB supplementation Microbial community <ul style="list-style-type: none"> Highest bacterial range weighted richness in the intestine of PHB-supplemented fish 	De Schryver <i>et al.</i> (2010b)
Giant freshwater prawn (<i>Macrobrachium rosenbergii</i>)	Larvae	Crystalline PHB alone or in combination with HUFA-rich lipid emulsion supplied via <i>Artemia</i> nauplii	Host <ul style="list-style-type: none"> Increased the survival and development of larvae fed PHB-supplied <i>Artemia</i> Microbial community <ul style="list-style-type: none"> Significantly low total bacteria and <i>Vibrio counts</i> in PHB-fed larvae 	Nhan <i>et al.</i> (2010)
Siberian sturgeon (<i>Acipenser baerii</i>)	Fingerlings	Crystalline PHB supplemented at varying levels in the diet	Host <ul style="list-style-type: none"> Improved the weight gain, SGR and survival in 2 % PHB supplemented diet 	Najdegerami <i>et al.</i> (2012)

			Microbial community <ul style="list-style-type: none"> Highest species richness at 2% PHB supplementation. PHB supplementation at 2 and 5 % stimulated <i>Bacillus</i> and other bacteria belonging to family of <i>Ruminococcaceae</i>. 		
Chinese mitten crab (<i>Eriocheir sinensis</i>)	Zoea 3 larvae	Crystalline PHB supplied via rotifers and <i>Artemia</i> nauplii	Host <ul style="list-style-type: none"> Enhanced the survival and growth of challenged and non-challenged larvae fed PHB-enriched livefeeds 	Sui <i>et al.</i> (2012)	
Nile tilapia (<i>Oreochromis niloticus</i>)	All males (25 ± 5 g)	Poly-beta-hydroxybutyrate-hydroxyvalerate (PHB–HV) extracted from <i>B. thuringiensis</i> A102 and supplemented at varying levels in the diet	Host <ul style="list-style-type: none"> Highest specific immune response in fish fed 5% PHB–HV supplemented diet Highest serum lysozyme activity in fish fed 5% PHB–HV supplemented diet Significant stimulation of serum peroxidase activity in fish fed 5% PHB–HV supplemented diet Dose-dependent increase of serum antiprotease activity Dose-dependent resistance against live virulent <i>A. hydrophila</i> 	Suguna <i>et al.</i> (2014)	
			Microbial community: N.R.		
Giant tiger prawn (<i>Penaeus monodon</i>)	Postlarvae (PL 1 and PL 30)	PHB-accumulating bacteria inoculated from sediment sample collected from shrimp culture pond	Host <ul style="list-style-type: none"> Higher survival of postlarvae fed PHB-supplemented diet in any developmental stages Higher growth of postlarvae fed PHB-supplemented diet in any developmental stages Higher survival of postlarvae fed PHB-supplemented diet after exposure to a pathogenic challenge Trend of higher survival in PL 30 after exposure to lethal dose of ammonium chloride 	Laranja <i>et al.</i> (2014)	
			Microbial community: N.R.		
Giant freshwater prawn	Larvae	Amorphous PHB supplied	Host	Thai <i>et al.</i> (2014)	

<i>(M. rosenbergii)</i>		via <i>Artemia</i> nauplii	<ul style="list-style-type: none"> Increased the survival and larval development in larvae fed PHB-supplied <i>Artemia</i> Increased the survival in challenged larvae fed PHB-enriched <i>Artemia</i> 	
Microbial community: N.R.				
Chinese mitten crab (<i>E. sinensis</i>)	Zoea 2 until megalopa stage	Crystalline PHB supplied via rotifers and <i>Artemia</i> nauplii	Host <ul style="list-style-type: none"> Enhanced the survival, larval development in larvae fed PHB-enriched livefeeds Enhanced the tolerance to osmotic stress in larvae fed PHB-enriched livefeeds 	Sui <i>et al.</i> (2014)
Microbial community: N.R.				
Siberian sturgeon (<i>A. baerii</i>)	Larvae	Crystalline PHB alone or in combination with HUFA-rich lipid emulsion supplied via <i>Artemia</i> nauplii	Host <ul style="list-style-type: none"> Decreased the growth in treatments either consisting of PHB or PHB + HUFA Decreased the survival in treatments either consisting of PHB or PHB + HUFA after exposure to salinity and ammonia stress tests Increased the whole-body lipid content in treatments either consisting of PHB or PHB + HUFA Decreased the contents of total saturated, monoenic, n-3 and n-6 LCFAs in treatments either consisting of PHB or PHB + HUFA High pepsin activity in treatments either consisting of PHB or PHB + HUFA Suppressed the amylase activity in treatments either consisting of PHB or PHB + HUFA 	Najdegerami <i>et al.</i> (2015)
Microbial community				
			<ul style="list-style-type: none"> Highest changes in gut microbial community in treatment consisting of PHB + HUFA 	
Blue mussel (<i>Mytilus edulis</i>)	Larvae	Crystalline and amorphous PHB supplemented in algal	Host <ul style="list-style-type: none"> Growth and metamorphosis were not improved in larvae fed 	Hung <i>et al.</i> (2015)

		diet	<p>PHB-supplemented algal diet</p> <ul style="list-style-type: none"> • Increased the survival in larvae fed amorphous PHB-supplemented algal diet • Addition of PHB degrading bacterial isolates did not improve outcome of PHB 	
			<p>Microbial community</p> <ul style="list-style-type: none"> • No relationship between changes in larvae-associated microbiota and improved survival following PHB treatment 	
<i>A. franciscana</i>	Newly-hatched	Crystalline PHB	<p>Host</p> <ul style="list-style-type: none"> • Highest survival of <i>Vibrio</i>-challenged <i>Artemia</i> when PHB was dosed at 100 mg L⁻¹ • Increased the production of Hsp70 in <i>Vibrio</i>-challenged <i>Artemia</i> • Generation of protective innate immune response (e.g. prophenoloxidase and transglutaminase immune systems) in <i>Vibrio</i>-challenged <i>Artemia</i> <p>Microbial community: N.R.</p>	Baruah <i>et al.</i> (2015)
Nile tilapia (<i>O. niloticus</i>)	Juveniles	Crystalline PHB supplemented at varying levels in the diet and amorphous PHB supplied via <i>Artemia</i> nauplii	<p>Host</p> <ul style="list-style-type: none"> • Trend of increasing weight gain in PHB-supplemented tilapia juveniles • Increased the SGR at 5 % PHB supplementation • Higher survival in larvae fed PHB-enriched <i>Artemia</i> after exposure to the pathogen <i>E. ictaluri</i> gly09R • Activities of trypsin, amylase and pepsin were not influenced by PHB-supplementation • Increased the lipase activity at high levels of PHB supplementation • Total protein content was not influenced by PHB supplementation • Increased the whole-body lipid content in PHB-supplemented juveniles • Increased the contents of saturated and monounsaturated LCFAs at high levels of PHB supplementation 	Situmorang <i>et al.</i> (2016)

- Contents of 20:5(n-3), 22:6(n-3) and total n-3 FAs so as the n-6/n-3 ratio were not influenced by PHB supplementation

Microbial community: N.R.

N.R. = not reported.

1.6.6.1 Energy-delivering effects of PHB

The energy-delivering effects of PHB were first demonstrated by Defoirdt *et al.* (2007b) using axenic *Artemia* nauplii. A significantly higher survival was obtained in case of *Artemia* nauplii supplemented with PHB as compared to starved axenic *Artemia* nauplii. In juvenile European sea bass *Dicentrarchus labrax*, survival at the end of a 6-week feeding trial was higher for juveniles fed 100 % PHB as compared to nonfed juveniles (De Schryver *et al.* 2010b).

The mechanism behind the energy-delivering effects of PHB in aquatic animals remained unknown but clinical trials and application in terrestrial animals such as rats (Klein *et al.* 2010) and pigs (Mulier *et al.* 2012) have shown that β -hydroxybutyrate (β -HB), the monomer and expected degradation product of PHB, is an important substrate for energy (Schade & Eaton 1979). Shivakumar (2013) has shown through paper chromatography that β -HB is the major end product of PHB hydrolysis. In humans, during normal physiological conditions, β -HB is naturally present in the serum and makes up more or less 75 % of the ketone bodies (KB) (Kimura *et al.* 1985). The term “KB” refers to the three molecules: β -HB, the dehydrogenated counterpart acetoacetate (AcAc), and the decarboxylated AcAc, acetone. KBs are produced by the liver and are used as an energy source when glucose is not readily available (Laffel 1999). Trials conducted on the mammalian brain suggested that β -HB can replace glucose as the brain's primary fuel during starvation (Owen *et al.* 1967). The utilization of β -HB in the mammalian brain requires conversion of β -HB to AcAc by mitochondrial D- β -hydroxybutyrate dehydrogenase. Next, the succinyl-CoA:3-oxoacid-CoA transferase (also known as SCOT)

converts AcAc to acetoacetyl-CoA. The mitochondrial thiolase cleaves the acetoacetyl-CoA into two molecules of acetyl-CoA which is further oxidized and its energy transferred as electrons to NADH and flavin adenine dinucleotide (FADH₂) in the TCA cycle (Figure 1.7) and further to adenosine triphosphate (ATP) in the mitochondrial respiratory chain. β -HB along with AcAc are also transported by the blood to the extrahepatic tissues, where they are oxidized via the TCA cycle to provide the energy required by tissues such as skeletal and heart muscle and the renal cortex (Dedkova & Blatter 2014).

The presence of β -HB in aquatic organisms was also illustrated in a number of studies. Studies dealing on elasmobranch fishes used enzyme activity measurements to demonstrate their reliance on ketone bodies (mainly β -HB and AcAc) as metabolic fuels. In the starved spiny dogfish shark *Squalus acanthias*, a tissue-specific activity of β -hydroxybutyrate dehydrogenase was observed in the order of rectal gland > anterior stomach > intestine > kidney > liver > posterior stomach > brain > muscle, gill and esophagus (Walsh *et al.* 2006). The serum β -HB concentrations and ranges for various species of sharks were studied by Watson & Dickson (2001). The highest β -HB concentration in the serum was observed in shortfin mako shark *Isurus oxyrinchus* (0.978 $\mu\text{mol mL}^{-1}$) while the lowest was observed in the blacktip shark *Carcharhinus limbatus* (.128 $\mu\text{mol mL}^{-1}$).

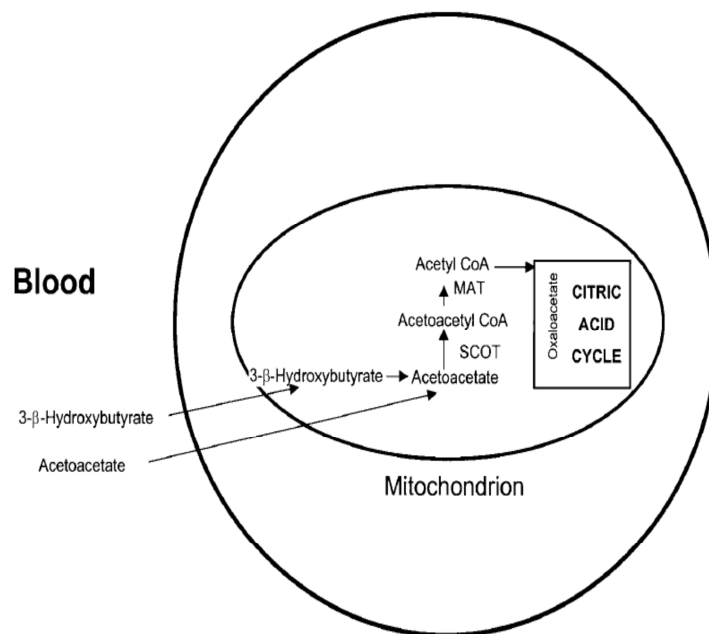


Figure 1.7 Utilization of β -HB and AcAc in the mammalian brain during prolonged starvation (Laffel 1999).

In the holocephalans, the activity of D- β -hydroxybutyrate dehydrogenase in the kidney, heart and liver of the spotted ratfish *Hydrolagus colliei* was investigated by Speers-Roesch *et al.* (2006). They found D- β -hydroxybutyrate dehydrogenase at a concentration of 0.61, 1.51 and 1.23 $\mu\text{mol min}^{-1} \text{g body weight}^{-1}$, respectively. D- β -hydroxybutyrate dehydrogenase was also detected in white muscle at a concentration of 0.04 $\mu\text{mol min}^{-1} \text{g body weight}^{-1}$. In teleost fishes, however, studies dealing with the presence of ketone bodies showed varying results. Segner *et al.* (1997) did not detect the presence of β -HB in both fed and starved carp *Cyprinus carpio* inspite of low activities of hepatic 3-hydroxybutyrate dehydrogenase. Zammit & Newsholme (1979) found very low concentrations of β -HB ($< 0.001 \mu\text{mol mL}^{-1}$) in the plasma of seabass *D. labrax*, red mullet *Mullus sermuletus* and mackerel *Scombrus scombrus*. Leblanc & Ballantyne (2000)

detected the presence of both D- β -hydroxybutyrate dehydrogenase (in the mitochondria of the liver) ($0.67 \mu\text{mol min}^{-1} \text{ g body weight}^{-1}$) and L- β -hydroxybutyrate dehydrogenase (cytosol in kidney) ($0.62 \mu\text{mol min}^{-1} \text{ g body weight}^{-1}$) in fed goldfish *Carassius auratus*.

The presence of β -HB in crustaceans was also evaluated. Graszynski (1970) did not detect the presence of β -hydroxybutyrate dehydrogenase in any organ of the cray fish *Ortonectes limosus* and thus no β -HB was seen in the haemolymph. Wang *et al.* (2014b) reported the existence of acyl-CoA oxidase in the hepatopancreas of gazami crab *Portunus trituberculatus* that is necessary for the oxidation of fatty acids, generating aldehydes and ketones. They also found mitochondrial-like D- β -hydroxybutyrate dehydrogenase. Beis *et al.* (1980) and Tsokos *et al.* (1983) have indirectly shown that the hepatopancreas of spiny lobster *Panulirus argus*, common lobster *Homarus vulgaris* and green crab *Carcinus maenas* can metabolise ketone bodies. The first direct evidence on the presence of β -HB in crustaceans was demonstrated by Weltzien *et al.* (2000). They found β -HB in newly hatched *Artemia* nauplii at a concentration of $0.61 \text{ nmol ind}^{-1}$ and this concentration increased to $1.0 \text{ nmol ind}^{-1}$ after a starvation period of 28-30 h. They suggested that β -HB is used as fuel in the developing *Artemia*.

1.6.6.2 Growth-promoting effects of PHB

The beneficial effects of PHB on the growth of aquatic farmed hosts were also evaluated. De Schryver *et al.* (2010b) measured the fish weight gain of juvenile European sea bass fed with crystalline PHB replaced at 0, 2, 5, 10 and 100 % in the diet. Significantly highest weight gain of 271 % was observed for juveniles fed 5 % PHB. Najdegerami *et al.* (2012) also examined the growth promoting effects of PHB in Siberian

sturgeon *Acipenser baerii* fingerlings fed with 0, 2 and 5 % crystalline PHB supplementation in the diet. The weight gain at the end of the 10-week feeding trial was highest in treatment fed a diet containing 2 % PHB although not significantly different from the other treatments. In sturgeon larvae, however, PHB delivered via *Artemia* nauplii did not improve the weight gain (Najdegerami *et al.* 2015). Situmorang *et al.* (2016) found a trend of higher final weight in Nile tilapia *Oreochromis niloticus* juveniles fed various levels of crystalline PHB in the diet. Fish weight at the end of the 28-day feeding trial was 218, 284, 269 and 258 mg for fish fed diets containing no PHB or PHB-supplemented diets at 5, 25 and 50 g kg⁻¹, respectively. Again, these values were not statistically different from each other. From all these studies, it can be concluded that the growth promoting effects of PHB in fish are stage and species dependent.

The growth promoting effects of PHB in crustaceans have also been evaluated recently. The larval stage index (LSI) of giant freshwater prawn *Macrobrachium rosenbergii* larvae significantly improved when these were fed crystalline PHB delivered via *Artemia* nauplii (Nhan *et al.* 2010). Similarly, Sui *et al.* (2012) found a significantly higher LSI in Chinese mitten crab *Eriocheir sinensis* larvae fed PHB delivered via rotifers and *Artemia* as compared to larvae fed non-enriched rotifers and *Artemia*. The growth-promoting effects of supplementing amorphous PHB was also tested. A significantly improved growth was observed in case PHB-accumulated bacteria were supplied in the diet of tiger shrimp *P. monodon* postlarvae and this seemed to correspond with the levels of PHB contained in the bacterial cells (Laranja *et al.* 2014). Similar findings were reported by Thai *et al.* (2014) who tested varying levels of *A. eutrophus* containing either

10 % or 80 % PHB on DW delivered via *Artemia* to giant freshwater prawn *M. rosenbergii* larvae. The LSI of *Macrobrachium* larvae fed *Artemia* enriched with *A. eutrophus* containing 10 % PHB was not significantly different from larvae fed non-enriched *Artemia*. However, a significantly higher LSI was observed for larvae fed *Artemia* enriched with *A. eutrophus* containing 80 % PHB. From these experiments, the application of bacteria containing high levels of PHB is recommended.

1.6.6.3 Protective agent against pathogenic bacteria

The potential beneficial effects of PHB as antimicrobial agent in aquaculture have recently gained considerable attention. The earliest study to test the potential of PHB to provide protection against pathogenic *V. campbelii* LMG21363 was conducted by Defoirdt *et al.* (2007b). In their *in vivo* test using *Artemia* nauplii, PHB was found to significantly enhance the survival of the nauplii and this result was more pronounced at higher concentrations of PHB.

The anti-pathogenic effect of PHB was also tested in other aquatic organisms. In fish, enrichment of *Artemia* nauplii with PHB-accumulated *A. eutrophus* containing 70 % PHB on DW brought significant protection in Nile tilapia juveniles against *Edwardsiella ictaluri* (Situmorang *et al.* 2016). In crustaceans, Nhan *et al.* (2010) found significantly lower total TCBS (Thiosulfate-citrate-bile salts-sucrose agar) count (presumed vibrios) of 1.6×10^2 larva⁻¹ in the gut of *Macrobrachium* larvae fed crystalline PHB enriched *Artemia* as compared to larvae fed non-enriched *Artemia* with total TCBS count of 13.3×10^2 larvae⁻¹. The survival of *V. anguillarum* challenged Chinese mitten crab larvae fed PHB

enriched *Artemia* offered either 24 h before challenge (26 % survival), simultaneously with challenge (19 % survival) or 24 h after challenge (12 % survival) at 16 days after hatching was significantly higher than challenged larvae fed non-enriched *Artemia* (0.6 %). Moreover, the fact that the larvae fed PHB enriched *Artemia* 24 h before challenge has significantly highest survival suggested that earlier delivery of PHB results in better protection (Sui *et al.* 2012). The protective capacity of PHB-accumulated bacterial isolates was also demonstrated in a number of studies (Laranja *et al.* 2014; Thai *et al.* 2014). Halet *et al.* (2007) have shown that addition of either non-treated or pasteurized, dried or frozen/thawed PHB-accumulated isolates (PHB2) containing up to 32 % PHB on cell DW to the culture water of *Artemia* before or 1 day after the addition of *V. campbellii* LMG 21363 significantly protected the *Artemia*.

The antipathogenic effect of PHB is not yet understood. However, it is assumed that PHB is biologically degraded into β -HB upon ingestion (Defoirdt *et al.* 2007a). The monomer could then exhibit growth inhibition towards certain pathogens like other SCFAs do (Defoirdt *et al.* 2007b). SCFAs exhibit antibacterial properties depending on the status of the organisms and the physicochemical characteristics of the external environment (Ricke 2003), mainly pH (Defoirdt *et al.* 2009). The undissociated and dissociated forms of fatty acids are in an equilibrium that depends on pH and the pK_a of the fatty acid (according to the Henderson–Hasselbach equation). The entry of SCFAs into bacterial cells occurs in the undissociated form by a diffusion process (Cherrington *et al.* 1991). Once internalised into the pH neutral cytoplasm, SCFAs dissociate into anions and protons (Kashket 1987). Generation of anions and protons presents a problem for bacteria that

must maintain a constant pH in the cytoplasm in order to sustain functional macromolecules. Consequently, export of excess protons requires consumption of cellular ATP and may result in depletion of cellular energy intended for growth and/or virulence (Figure 1.8).

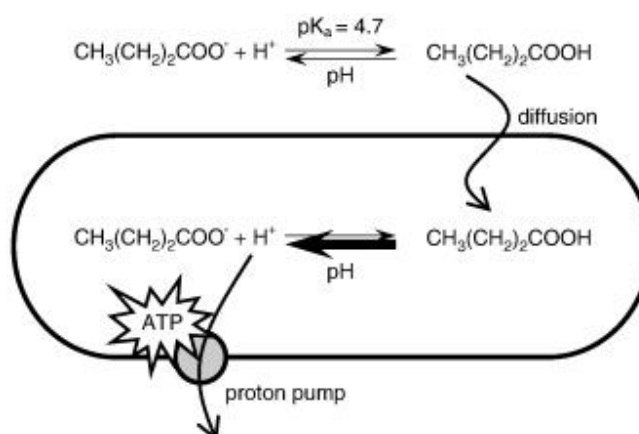


Figure 1.8 Mechanism of antipathogenic activity of SCFAs (taking butyric acid as example). The fatty acids pass the cell membrane in their undissociated form and dissociate in the cytoplasm. As a consequence, the cells have to spend energy to export excess protons (Defoirdt *et al.* 2009).

Few studies have also indicated the capacity of PHB to act as immunostimulant. In an *in vitro* experiment conducted on Mossambique tilapia *O. mossambicus* (25 g), Suguna *et al.* (2014) found stimulation of serum lysozyme activity, total peroxidase activity and antiprotease activity in fish fed a diet containing either 1 %, 3 % or 5% (w/w) of PHBV polymer extracted from *Bacillus thuringiensis* A102 strain as compared to fish fed non PHBV supplemented diet. Supplementing crystalline PHB at a concentration of 100 mg L⁻¹ was also found to induce the production of heat shock protein 70 (Hsp70) in *Vibrio*-

challenged *Artemia* nauplii. Expression of prophenoloxidase (*proPO*) gene, the immune effector of PO having important roles in cuticular melanization, sclerotisation, wound healing, encapsulation and eventual killing of pathogens, was also induced by PHB at this concentration (Baruah *et al.* 2015).

1.7 Rationale and objectives of the study

The potential of the bacterial storage compound PHB has been tested in several aquatic farmed species. Growth promotion, improved robustness against adverse environmental conditions and enhanced resistance against pathogens are but of the few documented beneficial effects of PHB (Chapter 1). Despite these reports, very few studies have been done to explore its actual mechanisms of action. In addition to this, very few attempts are made to explore the potential beneficial effects of PHB on penaeid shrimp, being the most important internationally traded fishery commodity in terms of value and the most valuable fishery export for many tropical developing countries (FAO 2009; Bondad-Reantaso *et al.* 2012).

To test the energy-delivering effects of PHB in penaeid shrimp, the survival of PHB-supplemented postlarval tiger shrimp and its robustness against adverse environmental conditions are explored in Chapter 2. The capacity of PHB to promote growth in penaeid shrimp is also investigated by measuring the larval stage index (LSI) of differentially fed postlarvae. Earlier reports have regarded PHB as new biocontrol agent in aquaculture, and therefore its resistance enhancing effects in *Vibrio*-challenged postlarvae are measured. Finally, supplementing HUFA-rich lipid emulsion to PHB is endeavoured to

determine whether this strategy could improve the putative effects of PHB as earlier reports have mentioned that PHB lacks the essential nutrients required for growth, which are present in lipid emulsion.

To test the hypothesis that PHB influences lipid metabolism in aquatic farmed species, the whole body lipid contents and fatty acid profiles of crustacean model species *Artemia* sp. supplied with dietary PHB either in crystalline or amorphous form are examined in Chapter 3. The addition of the bacterial PHB degrader *Comamonas testosteroni* was earlier shown to increase the protective effects of PHB, and it is therefore investigated what could be the effect of co-supplying dietary PHB in crystalline or amorphous form with *C. testosteroni* on the lipid and fatty acid parameters of *Artemia* nauplii. Finally, earlier findings in literature have mentioned that co-supplementing PHB with dietary nutrients rich in HUFAs resulted in best overall performance of tested aquatic species. This strategy is therefore tested to determine whether this could also result in improved whole body lipid contents and fatty acid profiles of *Artemia* nauplii.

Earlier findings employing the use of ^{13}C stable isotope analysis in fish species have shown that PHB is assimilated and distributed in different organs following ingestion and gastrointestinal degradation. However, there have been no reports described for crustaceans. To test this, the ^{13}C signal of two crustacean species, postlarval whiteleg shrimp *L. vannamei* and *Artemia* sp., offered ^{13}C -labeled *Ralstonia eutropha* DSM545 containing 75 % PHB on dry weight is investigated in Chapter 4. Considering the hypothesis that PHB and/or its degradation product β -HB is used as energy source in aquatic farmed species, the assimilation of PHB in postlarval *L. vannamei* fed a basal diet

containing either low- or high level of dietary fats is investigated to determine whether a low-fat diet could result in lower PHB assimilation or a high-fat diet could result in high PHB assimilation in the tissues of crustaceans. Finally, the ^{13}C signal of different fatty acid groups in postlarval *L. vannamei* fed a diet supplemented with ^{13}C -labeled *R. eutropha* DSM545 is also examined to determine whether PHB is assimilated in the structural components of crustaceans.

The use of artificial substratum has been reported to enhance the growth and survival of penaeid shrimp, and therefore the exploration of a totally new approach to use PHB in penaeid shrimp culture, namely the use of PHB-based bioplastics as artificial substratum in penaeid culture systems, is investigated in Chapter 5. Its potential beneficial effects in improving robustness against adverse environmental conditions and resistance against infection by *Vibrio* sp. are also evaluated. Literature reports have also mentioned of the contributions of artificial substratum in improving water quality in shrimp culture systems and thus, the capacity of PHB substratum to maintain good water quality as well as its overall attractiveness towards postlarval penaeid shrimp are also considered.

Finally, in Chapter 6, a general discussion of the results obtained in these studies is provided including recommendations for future research.

Chapter 2

Poly- β -hydroxybutyrate (PHB) enriched *Artemia* sp. for tiger shrimp

***Penaeus monodon* larviculture**

Chapter 2

POLY- β -HYDROXYBUTYRATE (PHB) ENRICHED *Artemia* sp. FOR TIGER SHRIMP *Penaeus monodon* LARVICULTURE

Gladys Ludevese-Pascual¹, Joseph Laranja^{1, 2}, Edgar Amar², Patrick Sorgeloos¹, Peter Bossier^{1*} & Peter De Schryver^{1*}

¹ Laboratory of Aquaculture & *Artemia* Reference Centre, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University Rozier 44, B-9000 Gent, Belgium

²Southeast Asian Fisheries Development Centre/Aquaculture Department (SEAFDEC/AQD), Brgy. Buyuan, Tigbauan Iloilo 5021 Philippines

*Shared senior authorship

Abstract

The beneficial effects of PHB as supplement in tiger shrimp *P. monodon* postlarvae using a short term enrichment strategy via *Artemia* were examined. The effects of co-supplementing with a lipid emulsion were also evaluated to determine whether it yielded an additional benefit. Results on the average weight and larval development were not significantly different among postlarvae fed the different dietary treatments indicating that PHB supplementation could not be used to stimulate growth in *P. monodon* postlarvae while such positive results have been reported in other aquaculture species. Nonetheless, significantly higher survival was obtained in postlarvae fed PHB-enriched *Artemia* irrespective of lipid enrichment. In addition, PHB increased the survival of the postlarvae

after exposure to a lethal dose of ammonium chloride. Lipid supplementation nullified this effect. The cumulative mortality of postlarvae subjected to a sublethal concentration of ammonium chloride for 24 h and subsequent exposure to pathogenic *Vibrio campbelli* showed that PHB but not lipids could effectively enhance the resistance of the postlarvae. Co-supplementing lipids even significantly decreased this outcome. Our study indicates that PHB supplementation increases the quality of larval *P. monodon* and their chance of surviving under adverse environmental conditions. The short-term co-supplementation with lipid emulsion did not add to these effects.

Redrafted after: Ludevese-Pascual G., Laranja J.L., Amar E., Sorgeloos P., Bossier P. & De Schryver P. (2016) Poly- β -hydroxybutyrate (PHB) enriched *Artemia* sp. for the giant tiger prawn *Penaeus monodon* larviculture. Aquaculture Nutrition. doi: 10.1111/anu.12410.

2.1 Introduction

Diseases are a major constraint to aquaculture production of invertebrates (Bachère 2003). Crustacean larvae are often exposed to stressful conditions making them susceptible to infections. In the shrimp aquaculture industry, bacterial diseases are often considered as major problems resulting in production and economic losses (Balakrishnan *et al.* 2011). Mainly *Vibrio* species have been associated with low survival in hatchery or grow-out conditions. The natural abundance of *Vibrio* spp., their multiplication rates and their ability

to adapt to environmental changes in shrimp culture ecosystems contribute to the problem (Saulnier *et al.* 2000).

Environmental parameters (e.g. temperature, pH, levels of dissolved oxygen, etc.) play an important role in disease susceptibility because they may influence the capacity of the immune system (Le Moullac & Haffner 2000). In addition, farming of aquatic animals commonly involves displacement from their natural habitat to an environment that is new and sometimes stressful. It also necessitates the use of feeds that are often unnatural or artificial and it requires culturing at stocking densities that are much higher than what is naturally occurring. These are conditions that can increase the chance for exposure to pathogens, can compromise defensive responses and can facilitate pathogen replication and disease transmission (Walker & Mohan 2009). Cheng & Chen (1998) found sub-optimal temperature and pH to increase mortality in fresh water prawn resulting from infection with *Enterococcus*-like bacteria.

A number of measures have been developed to mitigate the impact of diseases in the shrimp aquaculture industry. Common practices range from disinfection of the rearing water to the application of chemotherapy (e.g. antibiotics) (Smith *et al.* 2003). The latter has become undesirable since they promote the selection for antibiotic-resistance in both the target pathogenic bacteria, as well as in all other microorganisms present in the environment. The application of preventive approaches such as vaccines, immunostimulants, prebiotics and probiotics to enhance the shrimp's disease resistance is becoming increasingly important and will be essential for the further development of more sustainable aquaculture practices. Recently, an alternative approach was suggested

implying the use of the bacterial storage compound poly- β -hydroxybutyrate (PHB): i.e. β -HB, the monomer of PHB, is known to exhibit some antimicrobial, insecticidal, and antiviral activities (Tokiwa & Ugwu 2007), comparable to other short chain fatty acids (SCFAs) (Defoirdt *et al.* 2006). Disruption of the bacterial cell wall resulting in leakage, interference with nutrient transport, and altered energy or molecule synthesis are examples of less direct growth interfering effects associated with SCFA (Ricke 2003).

PHB and/or its degradation product β -HB can probably also be used as an energy source by crustaceans (Sui *et al.* 2014). Organic acids, mainly butyric acid, have also been mentioned as typical energy sources for the growth of intestinal epithelial cells (Biagi *et al.* 2007). In clinical trials and application in terrestrial animals such as rats (Klein *et al.* 2010) and pigs (Mulier *et al.* 2012), β -HB is reported to be naturally present in the serum and makes up more or less 75 % of the ketone bodies (KB). KBs are produced by the liver that are used as an energy source when glucose is not readily available (Laffel 1999). Defoirdt *et al.* (2007b) found a prolonged survival in case starved nauplii were supplied with PHB and an increased survival and growth in the case PHB was used as a feed additive. It remains, however, to be determined if the energy delivery effect of PHB may increase the robustness of crustaceans to counteract adverse rearing conditions. It is therefore the goal of this study to demonstrate the potential application of PHB for increasing the robustness of the tiger shrimp *Penaeus monodon* postlarvae during culture. The capacity of PHB to promote growth in penaeid shrimp is also investigated by measuring the larval stage index (LSI). Earlier reports have also regarded PHB as new biocontrol agent in aquaculture, and therefore its resistance enhancing effects in *Vibrio*-challenged postlarvae are described.

Finally, the effects of co-supplying PHB and lipid emulsion rich in highly unsaturated fatty acids (HUFAs) are endeavoured to determine whether this strategy could improve the putative effects of PHB as earlier reports have mentioned that PHB lacks the essential nutrients required for growth, which are present in lipid emulsion.

2.2 Materials and Methods

2.2.1 Test animals and experimental set-up

P. monodon larvae at late PZ stages (PZ2-3) were obtained from the Shrimp hatchery of the Southeast Asian Fisheries Development Centre/Aquaculture Department (SEAFDEC/AQD) (Tigbauan, Iloilo, Philippines). Larvae were transported to the laboratory using a double-layered plastic bag half filled with natural seawater and oxygenated before sealing. This bag was placed in one 25 L styrofoam box (36 cm long x 26 cm wide x 27 cm deep and 2.0 cm thickness). Upon arrival, larvae were acclimatized to laboratory conditions in 250 L cylindroconical fiberglass tank filled with 32 g L⁻¹ UV-treated seawater at 30 ± 2 °C for 1 week. During the acclimatization period, larvae were fed an algal mix mostly composed of *Chaetoceros* spp. obtained at the Larval Food Laboratory of SEAFDEC/AQD (Parado-Esteva *et al.* 1991). The overall status of the test animals was also examined. Randomly collected samples obtained from the same original batch were submitted to the Fish Health Section of SEAFDEC/AQD for diagnosis of white spot syndrome virus (WSSV) following the methods of Kimura *et al.* (1996) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) using the IQ2000TM IHHNV Detection System (Farming IntelliGene, Taiwan). The presence of monodon-type

baculovirus (MBV) using malachite green staining was also examined. Other morphological characteristics such as gut diameter (6th abdominal segment), muscle width, and rostral spine count (dorsal and ventral) were also evaluated to ensure a good overall health status of the larvae (Solis 1988).

At the end of the acclimatization period, larvae now at early mysis stage were randomly distributed and stocked at a density of 100 individuals L⁻¹ to the experimental set-up consisting of 10 L plastic tanks (31 cm long x 27 cm wide x 27 cm deep) filled with 8 L UV-treated seawater. The seawater used in the set-up was passed through a filtration system consisting of a series of filter cartridges (5, 10 and 15 µm) before passing through an ultraviolet filter at a capacity of approximately 30 L min⁻¹ (Mega Fresh, Taiwan). Water was changed every two days at 25 % of the water volume with fresh UV-sterilized seawater to remove waste. When changing the water, a fabricated siphon (Diplex polyvinylchloride hose) was used to carefully drain the water into a separate bucket to trap mysis that could be siphoned up as well. The rearing water was analyzed on a regular basis to determine the total ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen using JBL Test Kits (Neuhofen, Germany) and the concentrations of total ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were maintained below 0.2, 0.1, and 10.0 mg L⁻¹, respectively. Each experimental tank was supplied with air through an air diffuser to maintain dissolved oxygen above 5 mg L⁻¹. Water temperature and salinity measured using YSI 556 MPS Multiprobe System (Japan) averaged to 30 ± 2 °C and 32 g L⁻¹, respectively. A linear fluorescent lamp (40 W, Philips, USA) was used to give a daily light regime of 10 h light and 14 h dark.

2.2.2 Enrichment of *Artemia* nauplii

HIGH 5 *Artemia* sp. cysts (INVE Aquaculture, Thailand) were incubated at 5 g L⁻¹ in UV-sterilized sea water and allowed to hatch in a 30 L hatching tank for 24 h. Vigorous aeration was provided with illumination set at approximately 27 $\mu\text{E m}^{-2}\text{s}^{-1}$. At approximately 36 h incubation, *Artemia* nauplii (now *Artemia* Instar II) were washed with UV-sterilized seawater, transferred and stocked at a density of 100 nauplii mL⁻¹ in new seawater. *Artemia* instar II nauplii were enriched with either crystalline PHB particles (98 % poly- β -hydroxybutyrate - 2 % poly- β -hydroxyvalerate, Goodfellow, Huntingdon, England) at 1 g L⁻¹, standard lipid emulsion (ICES 30/0.6/C, Han *et al.* 2000) at 0.3 g L⁻¹ or the combination of crystalline PHB + ICES 30/0.6/C (at 1 g L⁻¹ and 0.3 g L⁻¹, respectively). Non-enriched *Artemia* nauplii were also maintained. PHB particles were UV-sterilized prior to utilization and sieved over a sterile 50 μm sieve. PHB particles bigger than 50 μm were not used during enrichment. The enrichment of the *Artemia* was carried out following a drip method whereby the enrichment products were initially suspended in separate bottles containing 200 mL of UV-sterilized seawater. Drips containing the enrichment suspensions were delivered to the enrichment tanks over a period of approximately 30 minutes through a narrow tube attached to the bottle and controlled via a valve. Bottles were provided with aeration to ensure constant mixing of the enrichment products. As opposed to the standard practice of enriching *Artemia* nauplii for 24 h, the differentially enriched *Artemia* Instar II were harvested after 6 h and thoroughly rinsed with UV-sterilized seawater to remove excess enrichment product.

2.2.3 Feeding trials

Enriched and non-enriched *Artemia* nauplii administered through the rearing water were used as live food for the tiger shrimp from mysis to early postlarval stages. The experiment included the following dietary treatments (4 replicates each):

- Non-enriched *Artemia* (Art - P - L)
- Lipid emulsion-enriched *Artemia* (Art - P + L)
- PHB-enriched *Artemia* (Art + P - L)
- PHB + Lipid emulsion-enriched *Artemia* (Art + P + L)

Feed was administered daily at 9:00 and 16:00 h *ad libitum* and the feeding trial lasted for 21 days.

2.2.4 Measured parameters

The survival of postlarvae was determined at the end of the feeding trial by counting the number of surviving postlarvae in each tank. The percentage survival at the end of the experimental trial was computed following the formula of Thompson & Bergersen (1991): $\text{Survival (\%)} = (X/N-A)*100$ where, X is the number of postlarvae present at the end of the feeding trial, N is the number of postlarvae at stocking (day 0) and A is the total number of postlarvae sacrificed for larval stage index determination. The mean weight within each replicate tank was determined by randomly collecting 20 postlarvae. These were sacrificed by immersion in ice cold seawater, blotted dry to remove adhering water and weighed individually on an analytical balance with 0.0001 g precision. Next, the mean weight for each treatment at the end of the experimental trial was computed as the mean of the average weights from the replicate tanks within each treatment. The

larval development was assessed as the larval stage index (LSI) determined at different time points. Ten postlarvae per experimental tank were randomly collected each day for the first 13 days and the larval stage of each sampled larva was determined according to the description of Motoh (1981) and assigned with a value: mysis I = 1, mysis II = 2, ... until postlarva PL3 = 6 (Millamena & Bangcaya 2001). The LSI was calculated as follows:

$$LSI = \frac{(\text{total number of mysis I} \times 1 + \text{total number of mysis II} \times 2 + \dots + \text{total number of postlarvae PL 3} \times 6)}{\text{Total number of larvae}}$$

2.2.5 Stress test

The survival of the postlarvae following exposure to a lethal dose of ammonium chloride was evaluated at the end of the feeding trial. A preliminary experiment (3 replicates) was performed with 50 untreated postlarvae of the same original batch to establish the concentration of ammonium chloride causing death to 50 % of the test animals after 24 h (LD₅₀) in seawater (with an average pH and water temperature of 8.2 and 30 °C, respectively). The LD₅₀ was computed using the method of Reed & Muench (1938). Preparation of test solution was carried out according to the methods described by Najdegerami *et al.* (2015).

The computed LD₅₀ of 143 mg ammonium chloride L⁻¹ was used in the stress test that was carried out according to the method of Alcaraz *et al.* (1999) with slight modifications. From 4 experimental tanks per treatment, 50 postlarvae were randomly collected and transferred to 4 containers containing 2 L UV-sterilized seawater and exposed for 24 h to the test solution. Air was provided into each container using an air

stone diffuser. Test animals were not fed 12 h prior to the test and during the exposure period. The number of dead postlarvae was recorded after 24 h of exposure. Death was assumed when larvae were immobile and showed no response to external stimulus such as picking or touching with forceps.

2.2.6 *Vibrio challenge test*

The survival of the postlarvae following exposure to a pathogenic challenge with *Vibrio campbellii* LMG21363 was evaluated. At the end of the feeding trial, 50 postlarvae were randomly collected from each tank replicate and subjected to a sublethal concentration of ammonium chloride (100 mg L^{-1}) for 24 h. Each tank was individually aerated by means of an airstone diffuser. After 24 h, postlarvae were gently collected and transferred to a new tank containing $10^7 \text{ cells mL}^{-1}$ of the pathogenic *V. campbelli* LMG21363. Preparation of the bacterial strain was done according to the method described by Defoirdt *et al.* (2007b). Postlarvae were given supplementary feeds all throughout the exposure period, however, no water exchange was administered. Cumulative mortality (%) was recorded every 24 h for 15 days.

2.2.7 *Statistical analysis*

All data were analyzed using two-way analysis of variance (two-way ANOVA) to identify statistically significant differences among the variables tested (PHB and lipid emulsion) as well as any interactions among them. When interaction was significant, a one-way ANOVA was used to compare means between different groups. A $P \leq 0.05$ was

chosen as the significance level. Data on percentages were arcsine transformed. All statistical analyses were performed using SPSS Statistical software v. 11.5.

2.3 Results

2.3.1 Test animals

The tests performed on WSSV and IHHNV on the *P. monodon* postlarvae indicated that the postlarvae were not infected with any of the viruses or that the viral load was lower than the detection limit. The larvae were also found negative for MBV occlusion bodies and muscular deformities were not detected. These results indicated that the mysis used in this experiment were physically fit for the experiment.

2.3.2 Measured parameters

The average weight (mg) and survival (%) of the postlarvae after the 21-day feeding trial are shown in Table 2.1. Results from the 2 x 2 factorial design showed that the Table 2.1 Weight (mg) and survival (%) of *P. monodon* postlarvae fed differentially enriched *Artemia* during the 21 day feeding trial. *P*-value in bold indicates significant differences ($P \leq 0.05$).

Parameters	<u>Non-PHB enriched</u>		<u>PHB enriched</u>		<u>P-value</u>		
	Non-lipid enriched (Art - P - L)	Lipid enriched (Art - P + L)	Non-lipid enriched (Art + P - L)	Lipid enriched (Art + P + L)	PHB	Lipid	PHB x Lipid
Mean weight	3.0 ± 0.2	4.2 ± 0.6	3.1 ± 0.2	3.2 ± 0.4	0.245	0.128	0.141
Survival	29.0 ± 4.5	33.4 ± 5.6	44.4 ± 2.5	37.6 ± 4.4	0.046	0.797	0.240

Values represent means ± SEM ($n = 4$).

interaction between PHB and lipid did not significantly affect the weight of the postlarvae ($F = 2.560$, $P = 0.141$). Similarly, PHB ($F = 1.528$, $P = 0.245$) and lipid treatments ($F = 2.757$, $P = 0.128$) alone did not significantly influence the weight of the postlarvae at the end of the feeding trial. The interaction between PHB and lipid on the survival of the postlarvae was not statistically significant ($F = 1.586$, $P = 0.232$). However, PHB treatment ($F = 4.993$, $P = 0.045$) significantly influenced the survival of the postlarvae with a mean survival of 48 ± 2 % in postlarvae fed PHB-enriched *Artemia* while this was 34 ± 3 % for postlarvae fed non PHB-enriched *Artemia*. Lipid treatment did not contribute to the survival of the postlarvae ($F = .076$, $P = 0.788$). The development of the postlarvae fed the different dietary treatments is shown in Figure 2.1.

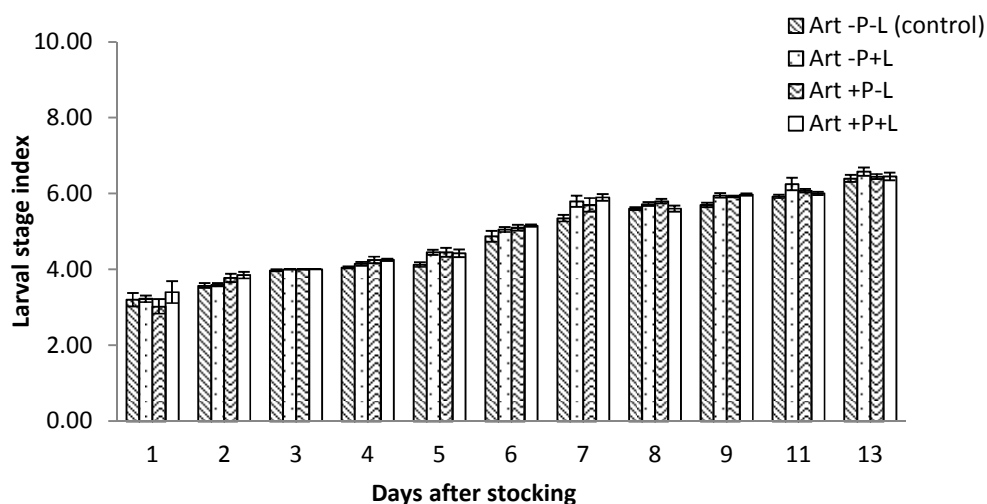


Figure 2.1 Larval stage index (LSI) of *P. monodon* postlarvae fed non-enriched (Art - P - L) and enriched *Artemia* (lipid emulsion-enriched (Art - P + L); PHB-enriched (Art + P - L); and PHB + lipid-emulsion enriched (Art + P + L)) during the 21 day feeding trial. Values represent means \pm SEM ($n = 4$). No significant differences were detected.

There was no significant interaction between PHB and lipid ($F = .860$, $P = 0.372$), and the PHB ($F = .158$, $P = 0.698$) or the lipid treatment ($F = .860$, $P = 0.372$) alone did also not significantly affect the development of the larvae.

2.3.3 Stress test

The protective effect of PHB and lipid supplementation in postlarval tiger shrimp after 24-h exposure to a lethal dose of ammonium chloride is shown in Figure 2.2.

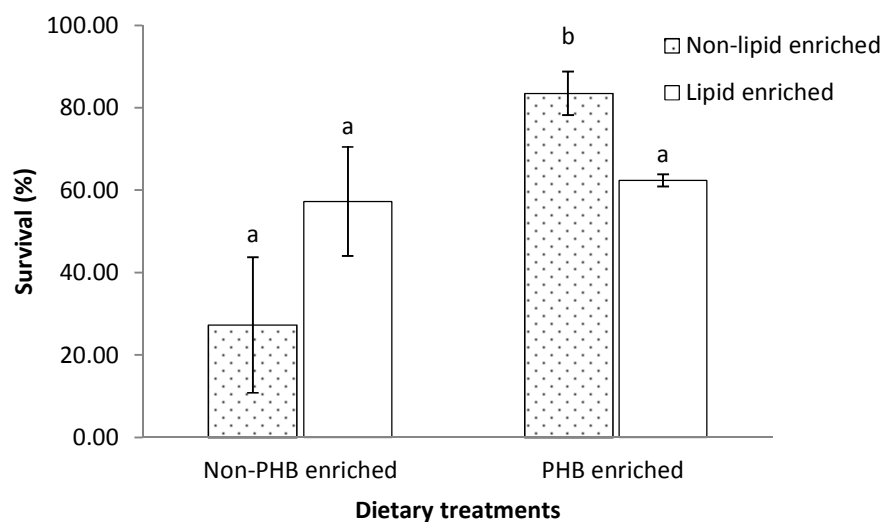


Figure 2.2 Survival (%) of *P. monodon* postlarvae fed differentially enriched *Artemia* for 21 days and subsequently exposed for 24 h to a lethal dose of ammonium chloride. Bars represent means \pm SEM ($n = 4$). Bars with different letters are significantly different ($P \leq 0.05$). Interaction between PHB and lipid was statistically significant ($F = 5.261$, $P = .043$) by which the PHB effect was nullified in combination with lipid enrichment.

There was a significant interaction between PHB and lipid affecting the survival of the postlarvae ($F = 5.261$, $P = 0.043$). Results from a one-way ANOVA have shown that in treatments without PHB, lipid supplementation did not significantly enhance the survival of the postlarvae ($P = 0.178$). PHB treatment alone, on the contrary, caused a significant increase in the survival of the larvae ($P = 0.018$). An additional lipid treatment nullified this effect.

2.3.4 *Vibrio* challenge test

Figure 2.3 shows the 15-day cumulative mortality (%) of *P. monodon* postlarvae after 24 h exposure in a sublethal concentration of ammonium chloride followed by bath challenge with *V. campbellii* LMG21363. The combined exposure to ammonium chloride followed by exposure to pathogenic *Vibrio* had a direct effect on the postlarvae as seen by the number of dead postlarvae after 24-h post challenge. Results from the 2 x 2 factorial design showed that the cumulative mortality of the postlarvae at 15 days post challenge was significantly affected by the interaction between PHB and lipid ($F = 9.597$, $P = 0.015$). Results from a one-way ANOVA have shown that PHB treatment significantly lowered the mortality of the postlarvae. However, in the presence of lipids, this effect was nullified. Lipid treatment alone did not influence the resistance of the postlarvae against the combined effects of sublethal ammonium chloride and pathogen bacteria ($P = 0.262$).

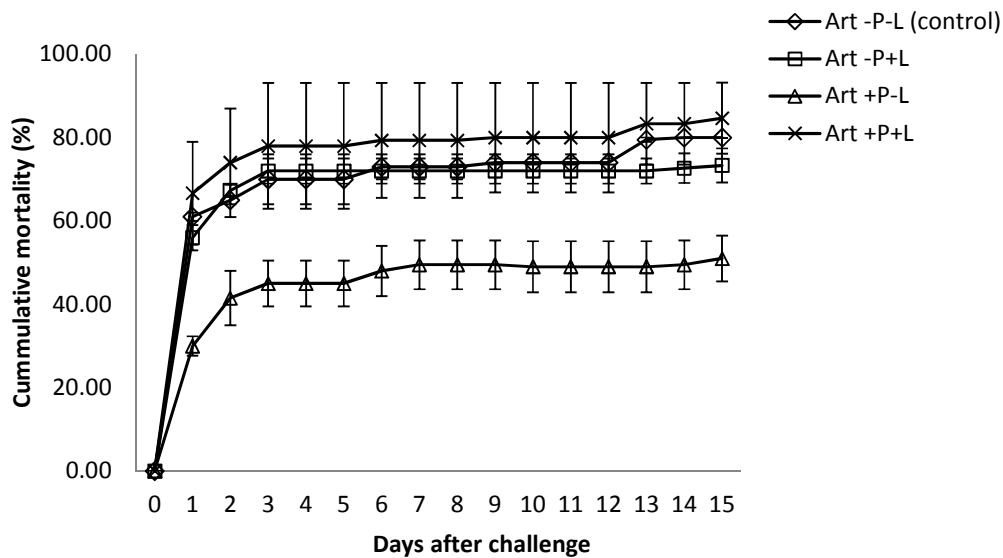


Figure 2.3 Cumulative mortality (%) of *P. monodon* postlarvae fed non-enriched (Art - P - L) and enriched *Artemia* (lipid emulsion-enriched (Art - P + L); PHB-enriched (Art + P - L); and PHB + lipid-emulsion enriched (Art + P + L)) followed by exposure to a sublethal dose of ammonium chloride for 24 h and subsequent challenge with pathogenic *V. campbellii* LMG21363. Values represent means \pm SEM ($n = 4$). Statistical analysis done at 15 days after challenge showed a significant interaction between PHB and lipid ($F = 9.597$, $P = .015$). PHB effect was nullified when combined with lipid enrichment.

2.4 Discussion

In various crustaceans and fish species, the application of the bacterial storage compound PHB as an alternative disease control approach has been tested (Defoirdt *et al.* 2007a; Defoirdt *et al.* 2009). Here, the potential of PHB encapsulated in *Artemia* nauplii in a short term enrichment strategy was demonstrated. Our findings indicated that although PHB supplementation could not be used to stimulate growth in *P. monodon* postlarvae, it

could effectively enhance the survival of the postlarvae. Most importantly, supplementation of PHB to the diet of *P. monodon* postlarvae resulted in a higher chance of surviving under adverse environmental conditions.

Supplementing lipid emulsion in addition to PHB in a short term enrichment strategy did not exhibit growth promoting effects in the postlarval *P. monodon* based on the comparable mean weight between postlarvae fed PHB-enriched and postlarvae fed PHB + lipid-enriched *Artemia*. This was also the case for the LSI. These findings are not in correspondence with the finding of Nhan *et al.* (2010) who showed that the LSI of *Macrobrachium rosenbergii* was significantly increased when larvae were fed 24-h enriched *Artemia* using a combination of PHB and lipid. Supplementation with PHB or lipid emulsion alone did not lead to a significant increase in growth and larval development either. Findings obtained by Sui *et al.* (2012) revealed that larval development of Chinese mitten crab *Eriocheir sinensis* larvae fed PHB-enriched *Artemia* was significantly improved. This indicates that the growth-promoting effects of PHB could be species dependent. Alternatively, the different gut microbial communities associated with different species could have caused this effect. Further research should be performed to investigate these aspects as well as focusing on the supplementation of different doses of PHB to *P. monodon* postlarvae including the quantification of the actual amount of PHB encapsulated in the *Artemia* and as such passed on to the next level in the food chain.

PHB supplementation alone brought significant increase in the survival of the postlarvae. This is in accordance with the studies conducted on other crustacean species (Nhan *et al.* 2010; Sui *et al.* 2012). Prolonged survival was also observed for PHB-fed

Artemia (Defoirdt *et al.* 2007b). These observations suggest that PHB and/or its degradation product β -hydroxybutyrate (β -HB) can be used as an energy source by crustaceans. Lipid supplementation did not enhance the survival of the *P. monodon* postlarvae. This result is not in accordance with the findings in literature. Bengtson *et al.* (1991) showed that feeding n-3 HUFA-enriched *Artemia* results in increased larval survival and growth in several *Penaeus* spp. and *M. rosenbergii*. Léger & Sorgeloos (1992) also demonstrated that in penaeid shrimp, feeding n-3 HUFA-rich *Artemia* during zoeal stages resulted in a better survival and growth in the later stages. While most *Artemia* enrichment trials employed long-term enrichment duration (e.g 24 to 48 h), the short-term lipid enrichment strategy used in this study may not have been sufficient to incorporate the lipids in the tissue of the *Artemia* resulting in low quality as larval food for the *P. monodon*. Dhont *et al.* (1991) stated that gradual and long-term enrichment has an advantage in that *Artemia* are able to accumulate lipids in their tissues in addition to the lipids accumulated in their gut. Lipids accumulated in the gut are more likely excreted before the *Artemia* is predated upon (Dhont, *pers. comm.*). The short-term enrichment with PHB in combination with lipids did not enhance the survival of the postlarval *P. monodon* either. It is hypothesized that supplementing lipids in addition to PHB during the short-term enrichment strategy decreased PHB uptake by the *Artemia* either due to a dilution effect by the lipids or the preference of the *Artemia* to take up one of the two enrichment products. As Makridis & Vadstein (1999) have reported food size selectivity of *A. franciscana* metanauplii, further research should be conducted to investigate preferential uptake by *Artemia* between PHB and lipid enrichment. In addition, the actual levels of

β -HB in differentially enriched *Artemia* should be investigated to determine whether a ketotic acidosis as mentioned by Weltzien *et al.* (2000) could have occurred specifically in treatment consisting of Art + P + L that resulted in an overall low performance of postlarval tiger shrimp.

Results on the stress test showed that PHB supplementation alone increased the survival of the *P. monodon* postlarvae indicating that PHB could enhance its robustness against adverse environmental condition. Lipid supplementation containing high levels of HUFA did not contribute to the robustness of the postlarvae. This is not in accordance with the findings of Martins *et al.* (2006) who reported that feeding n-3 HUFA-enriched *Artemia* to *Farfantepenaeus paulensis* larvae increased its survival and tolerance to ammonia. Cavalli *et al.* (2000) also showed higher ammonia tolerance in *Macrobrachium* larvae fed *Artemia* enriched with n-3 HUFA. These abovementioned studies employed 24-h *Artemia* enrichment. As it was previously mentioned, the short-term enrichment strategy used in this study may not be sufficient to incorporate the lipids in the tissue of the *Artemia* resulting in low quality food for postlarval *P. monodon*. Remarkably, the combined enrichment with PHB and lipids seemed to nullify the effect of PHB. This result supports our earlier statement that lipid enrichment could have potentially competed with the PHB uptake.

The resistance-enhancing effect of PHB against sublethal ammonium chloride concentration and subsequent exposure to pathogenic bacteria were also investigated. This approach was used as Gomez-Gil *et al.* (1996) stated that *Vibrio* spp. in general are opportunistic bacteria and mainly become a threat when the natural defence mechanisms

are suppressed. The significant reduction in mortality of postlarvae fed PHB-enriched *Artemia* confirms earlier results in literature on PHB as potential anti-microbial agent. PHB, according to Defoirdt *et al.* (2007b), may protect the host against infection by indirectly improving its overall fitness and directly, by inhibiting the growth of the pathogens. De Schryver *et al.* (2010a) have seen lower gut pH in European sea bass *Dicentrarchus labrax* juveniles fed high levels of dietary PHB suggesting an increase in the production of SCFAs in the gut. PHB was also found to protect the zoea of the Chinese mitten crab against pathogenic *Vibrio* (Sui *et al.* 2012). Suguna *et al.* (2014) also demonstrated a protective capacity of PHB against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. Protection against virulent pathogenic *Vibrio* was also obtained using PHB-accumulating bacteria isolated from activated sludge (Halet *et al.* 2007). PHB-accumulating bacteria supplied either through formulated shrimp diets (Laranja *et al.* 2014) or bioencapsulated in *Artemia* (Thai *et al.* 2014) protected the postlarvae of *P. monodon* and larvae of *M. rosenbergii*, respectively, against pathogenic *Vibrio*. Lipid supplementation alone or the combined supplementation of lipids and PHB did not result in an increased resistance of the postlarvae. The same explanations as given above (short-term enrichment and competition between PHB and lipids for uptake) are hypothesized to be the basis of these observations. It is evident that the substantial influence of lipids on the effect of PHB during a short-term enrichment strategy should be further investigated including the evaluation of nutritional composition of the differentially enriched *Artemia* to determine whether the short-term enrichment strategy employed in the present study could successfully incorporate the nutrients in the tissues of the *Artemia*.

In conclusion, efficient delivery of PHB via *Artemia* was achieved by a short-term enrichment strategy and this significantly improved the survival, robustness and resistance of the *P. monodon* postlarvae but not the growth. Supplementing dietary nutrients in the form of lipid emulsion in addition to PHB in a short-term enrichment strategy did not have an additional benefit. As report on literature have mentioned that co-supplying PHB with lipid emulsion employing a long-term enrichment strategy resulted in best overall culture performance of larval giant freshwater prawn (Nhan *et al.* 2010), long-term enrichment (i.e. 24-h) of *Artemia* nauplii with dietary nutrients rich in highly unsaturated fatty acids (HUFAs) followed by a PHB treatment during the last hours of the enrichment process should be investigated whether this strategy could also result in best overall culture performance of postlarval tiger shrimp.

Acknowledgment

This study was funded by the Flemish Interuniversity Council (VLIR). Peter DS is supported as a post-doctoral research fellow of the Research Fund – Flanders (FWO, Belgium).

Chapter 3

**The effects of poly- β -hydroxybutyrate (PHB) supplementation on the
lipid and fatty acid composition of crustaceans:**

***Artemia* sp. as model species**

Chapter 3

THE EFFECTS OF POLY- β -HYDROXYBUTYRATE (PHB) SUPPLEMENTATION ON THE LIPID AND FATTY ACID COMPOSITION OF CRUSTACEANS: *Artemia* sp. AS MODEL SPECIES

Gladys Ludevese-Pascual¹, Joseph Laranja^{1, 2}, Edgar Amar², Peter Bossier^{1*} & Peter De Schryver^{1*}

¹ Laboratory of Aquaculture & *Artemia* Reference Centre, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University Rozier 44, B-9000 Gent, Belgium

²Southeast Asian Fisheries Development Centre/Aquaculture Department (SEAFDEC/AQD), Brgy. Buyuan, Tigbauan Iloilo 5021 Philippines

*Shared senior authorship

Abstract

Lipids play a central role in the energy metabolism of aquatic animals. As earlier reports in fish species have suggested that the bacterial storage compound poly- β -hydroxybutyrate (PHB) could stimulate lipid digestion, here, three experiments were conducted to investigate whether dietary PHB could influence the lipid contents and the contents of four fatty acids (FAs) considered essential to penaeid shrimp, namely: linoleic acid (LOA; 18:2 (n-6)), linolenic (LNA; 18:3 (n-3)), eicosapentaenoic acid (EPA; 20:5 (n-3)) and docosahexaenoic acid (DHA; 22:6 (n-3)) using the crustacean model organism,

Artemia sp. In Experiment 1, supplying PHB in crystalline form (98 % poly- β -hydroxybutyrate - 2 % poly- β -hydroxyvalerate, Goodfellow, Huntingdon, England) or amorphous form (lyophilized cells of *Ralstonia eutropha* DSM545 containing 75 % of PHB on dry weight (DW)) at 100 mg PHB L⁻¹ for 48 h significantly increased the whole-body lipid contents of starved *Artemia* nauplii. Total lipid contents were 8.3 \pm 0.5 % and 9.3 \pm 0.7 % on DW in *Artemia* supplied with crystalline and amorphous PHB, respectively, while this was 6.5 \pm 0.2 % on DW in starved *Artemia*. Supplying PHB in amorphous form but not in crystalline form - significantly increased the contents of two essential polyunsaturated FAs (PUFAs) LOA and LNA from 3.2 \pm 0.2 and 15.4 \pm 1.2 mg g⁻¹ DW in starved *Artemia* to 3.9 \pm 0.1 and 20.4 \pm 0.9 mg g⁻¹ DW, respectively. But these FAs did not change based on total lipid indicating a non-preferential degradation of PUFAs in the presence of PHB. In Experiment 2, co-supplying dietary PHB either in crystalline or amorphous form with the PHB degrading bacterium *C. testosteroni* at 10⁶ cells mL⁻¹ for 24 h significantly increased the total lipid contents (12.1 \pm 0.6 % and 11.0 \pm 0.4 % on DW) as compared to starved *Artemia* (9.9 \pm 0.5 % on DW). This strategy also significantly increased the content of LOA from a value of 4.4 \pm 0.2 mg g⁻¹ DW for starved *Artemia* to a value of either 5.1 \pm 0.3 mg g⁻¹ DW or 5.1 \pm 0.1 mg g⁻¹ DW for *Artemia* co-supplied either crystalline or amorphous PHB and PHB degrading bacterium *C. testosteroni*, respectively. Similarly, content of LNA significantly increased from 23.1 \pm 1.1 mg g⁻¹ DW for starved *Artemia* to either 27.5 \pm 1.7 mg g⁻¹ DW or 27.6 \pm 0.7 mg g⁻¹ DW for *Artemia* co-supplied either crystalline or amorphous PHB and PHB degrading bacterium *C. testosteroni*, respectively. These FAs, however, did not change based on total lipid indicating the non-

preferential degradation of PUFAs in the presence of PHB as mentioned previously. The contents of two n-3 highly unsaturated FAs (HUFAs) eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) were not improved. Lastly, in Experiment 3, co-supplying dietary PHB in crystalline form with a HUFA-rich lipid emulsion at 0.6 g L⁻¹ did not significantly affect the nutritional quality of the lipid-supplied *Artemia* nauplii. Overall, this study demonstrated the lipid-saving effects of dietary PHB either in crystalline or amorphous form in the crustacean model organism, *Artemia* sp.

3.1 Introduction

In marine invertebrates, lipids are the predominant organic reserve (Sánchez-Paz *et al.* 2006). It has energetic and structural functions during embryonic and early larval development (Mourente *et al.* 1995). Aside from these, lipids are also known to act as energy reserve and are needed for the maintenance and integrity of cellular membranes, and serve as precursors for metabolic regulators such as prostaglandins, thromboxans and prostacyclins, hormones that promote maturation and molting (Davis 2005). Nutritional studies conducted in penaeid shrimp have placed most emphasis on the essential fatty acids (FAs) as they are necessary for normal growth (Liao & Liu 1989). Overall, four FAs are considered essential and these are: linoleic acid (LOA; 18:2 (n-6)), linolenic (LNA; 18:3 (n-3)), eicosapentaenoic acid (EPA; 20:5 (n-3)) and docosahexaenoic acid (DHA; 22:6 (n-3)) (FAO 2014). According to Kanazawa *et al.* (1979), the two n-3 highly unsaturated fatty acids (HUFAs) EPA and DHA have a greater growth promoting effect in shrimp than the n-3 polyunsaturated fatty acid (PUFA) LNA.

Recent findings by Situmorang (2015) have suggested that PHB could affect lipid metabolism by stimulating lipid digestion as indicated by the increasing activity of lipase in juvenile Nile tilapia *Oreochromis niloticus* fed a diet supplemented with crystalline PHB at 25 or 50 g kg⁻¹. Further, these authors found significantly higher total lipid contents of 28.3, 27.7 and 27.9 % on DW in juveniles fed diets containing 5, 25 and 50 g kg⁻¹ crystalline PHB, respectively, as compared to juveniles fed a non-PHB supplemented diet. The contents of two n-3 essential FAs EPA and DHA and the n-6/n-3 ratio, however, were not affected. Crystalline PHB either supplied alone via *Artemia* or in combination with a lipid emulsion rich in HUFAs significantly increased the whole-body lipid contents of Siberian sturgeon *Acipenser baerii* larvae (Najdegerami *et al.* 2015). However, contents of EPA and DHA increased only when larvae were supplied with the lipid emulsion, be it in combination with PHB or not. These authors also found a significant increase in n-3/n-6 ratio with a value of 4 for larvae fed non-enriched *Artemia* and a value of 4.6 for larvae fed PHB-enriched *Artemia*. This ratio further increased to 5.6 for larvae fed *Artemia* enriched with a combination of PHB and lipid emulsion. As the abovementioned findings were demonstrated in fish species and no attempts have been made in crustaceans so far, the present study aimed at investigating the influence of dietary PHB supplied either in crystalline or amorphous form on the lipid contents and the contents of four essential FAs in crustaceans using *Artemia* sp. as a model species. Literature have mentioned that the use of *Artemia* as model species in nutritional studies offered several advantages including the possibility to be cultured under axenic and gnotobiotic conditions (Verschuere *et al.* 1999), short generation time and availability of large quantities of cysts (Van Stappen 1996) and

finally, they are small-sized organism that can be easily cultured at high density (Soltanian 2007). The effects of co-supplying dietary PHB with the bacterial PHB degrader *Comamonas testosteroni* on the lipid contents and the contents of four essential FAs in *Artemia* are also examined as earlier reports have mentioned that addition of bacterial PHB degraded could increase the protective effects of PHB (Thai *et al.* 2015; Halet *et al.* 2007). Finally, the effects of co-supplying dietary PHB with HUFA-rich lipid emulsion are also investigated as earlier report in literature mentioned that PHB lacks essential nutrients required for growth, which are present in HUFA-rich lipid emulsion (Nhan *et al.* 2010).

3.2 Materials and Methods

3.2.1 Axenic Artemia culture

Artemia sp. cysts originating from the Great Salt Lake, Utah, USA (EG type; INVE Aquaculture NV, Belgium) were used in this experiment. Sterile and decapsulated *Artemia* cysts obtained via the decapsulation procedure described by Marques *et al.* (2005) were incubated at 1 g L^{-1} in a glass bottle containing 1 L filtered autoclaved seawater (FASW) and allowed to hatch under vigorous aeration and illumination set at approximately $27 \mu\text{E m}^{-2} \text{ s}^{-1}$. At 36 h of incubation, *Artemia* nauplii (now at the Instar II stage) were washed carefully with FASW on a $150 \mu\text{m}$ sieve and transferred at a density of $100 \text{ nauplii mL}^{-1}$ to sterile incubation bottles containing 1 L FASW. The axenic *Artemia* nauplii were subsequently treated depending on the treatments in the different experiments.

3.2.2 Experiment 1. PHB supplementation for *Artemia* nauplii: crystalline versus amorphous PHB

The effects of supplying either crystalline or amorphous PHB on the lipid contents and the contents of four essential FAs of *Artemia* nauplii was evaluated. The *Artemia* nauplii in the incubation bottles were either starved (control treatment, $n = 4$) or supplied with the following products ($n = 5$ per treatment):

- Crystalline PHB (Art + PHB_c)
- Amorphous PHB (Art + PHB_a)

The crystalline PHB (98 % poly- β -hydroxybutyrate - 2 % poly- β -hydroxyvalerate, Goodfellow, Huntingdon, England) and the amorphous PHB (produced according to Thai *et al.* (2015)) were supplemented at 100 mg PHB L⁻¹ in the incubation bottles. Prior to utilization, both PHB forms were suspended in a 50 mL erlenmeyer flask containing 30 mL FASW and sonicated using a Branson 1200 bath sonicator device (Connecticut, USA) for 5 mins with occasional shaking. Enrichment was performed for 48 h in 1 L incubation bottles containing 800 mL FASW equipped with a 0.20 μ m filter (Sartorius) on the aeration in- and outlet and placed under constant illumination (approximately 27 μ E m⁻² s⁻¹) at 28 °C. At the end of the enrichment period, the differentially enriched *Artemia* nauplii were washed with FASW on a 150 μ m sieve and transferred to 1.5 g L⁻¹ cellulose in FASW suspension for 8 h to purge enrichment particles from the gut of the nauplii (Niu *et al.* 2012). Subsequently, *Artemia* nauplii were harvested on a 200 μ m sieve and thoroughly washed with FASW to remove adhering cellulose

particle. The harvested nauplii were concentrated in 40 mL brown storage vials and stored immediately at - 20 °C until lipid and fatty acid analyses.

3.2.3 Experiment 2. PHB supplementation for *Artemia* nauplii: co-supplying PHB with the bacterial PHB degrader *C. testosteroni*

The effects of co-supplying PHB with the bacterial PHB degrader *C. testosteroni* on the lipid contents and the contents of four essential FAs of *Artemia* nauplii was investigated. The *Artemia* nauplii in the incubation bottles were either starved (control treatment, $n = 4$) or supplied with the following products ($n = 5$ per treatment):

- Crystalline PHB and bacterial PHB degrader (Art + PHB_c + C_t)
- Amorphous PHB and bacterial PHB degrader (Art + PHB_a + C_t)
- Bacterial PHB degrader (Art + C_t)

The crystalline and amorphous PHB were treated and supplied as described for Experiment 1 while the bacterial PHB degrader *C. testosteroni* LMG19554 was grown overnight in marine broth (Difco Laboratories, Detroit, USA), harvested by centrifugation at 8 000 rpm for 5 mins and added to the culture water at 10^6 cells mL⁻¹. The enrichment was performed for 24 h in 1 L incubation bottles containing 800 mL FASW equipped with a 0.20 µm filter (Sartorius) on the aeration in- and outlet and placed under constant illumination (approximately 27 µE m⁻² s⁻¹) at 28 °C. Subsequently, the differentially enriched *Artemia* nauplii were harvested, treated and stored as described for Experiment 1.

3.2.4 Experiment 3. PHB supplementation for *Artemia*: co-supplying PHB with a HUFA-rich lipid emulsion

The effects of co-supplying crystalline PHB with a HUFA-rich lipid emulsion on the lipid contents and the contents of four essential FAs of *Artemia* nauplii was investigated. The *Artemia* nauplii in the incubation bottles were either starved (control treatment, $n = 3$) or supplied with the following products ($n = 3$ per treatment):

- Crystalline PHB only (Art + PHB_c - L)
- Lipid emulsion only (Art - PHB_c + L)
- Crystalline PHB and lipid emulsion (Art + PHB_c + L)

The crystalline PHB was treated and supplied as described for Experiment 1 while the lipid emulsion (A1 DHA SELCO, Inve Group, Belgium) was supplied at 0.6 g L⁻¹. The enrichment was performed as described for Experiment 2. Differentially enriched *Artemia* nauplii were harvested, treated and stored as described for Experiment 1.

3.2.5 Total lipid content and fatty acid composition

All samples from the abovementioned experiments were analyzed for total lipid contents and the contents of different fatty acid groups. In the present study, however, only the results on four fatty acids considered essential to penaeid shrimp are presented. Dry weight of the *Artemia* samples was determined by oven drying 200 mg of the wet sample for 4 h at 103 °C. Total lipid was extracted and prepared according to the method described by Folch *et al.* (1957) and expressed as percentage on dry weight (% on DW). Fatty acid analysis was performed following the method of Lepage & Roy (1984) by direct acid-

catalyzed transesterification of the *Artemia* samples (approximately 200-500 mg on wet weight) without prior lipid extraction. An internal standard 20:2 (n-6) was added before the reaction and fatty acid methyl esters (FAMES) were extracted using hexane. Quantitative determination was done using a Chrompack CP9001 gas chromatograph equipped with an autosampler (Curé *et al.* 1996). The results are expressed as mg FAMES per gram lipid (mg g^{-1} lipid) and mg FAMES per gram dry weight (mg g^{-1} DW).

3.2.6 Statistical analysis

Descriptive statistics were used to assess normality of the data. When the assumption of normality was achieved, data in Experiment 1 and 2 were subjected to one-way analysis of variance (ANOVA) followed by a Duncan's multiple range post-hoc test to identify significant differences among treatment means. When data did not meet normality or variances were not homogenous after various transformation, the non-parametric Kruskal-Wallis test followed by a Mann-Whitney *U*-test was used. In Experiment 3, two-way ANOVA was used to identify statistically significant differences among the variables tested (PHB, lipid emulsion and its interaction) followed by a Bonferroni multiple comparison test. The alpha level was set a $P \leq 0.05$ for all statistical tests. All statistical analyses were performed using SPSS Statistical software v. 11.5.

3.3 Results

3.3.1 Experiment 1. PHB supplementation for *Artemia* nauplii: crystalline versus amorphous PHB

Table 3.1 shows the effects of PHB on the lipid contents and the contents of four essential FAs in *Artemia*. Supplying dietary PHB either in crystalline (Art + PHB_c) or amorphous (Art + PHB_a) form significantly increased the whole-body lipid contents of the *Artemia* nauplii as compared to the control *Artemia* nauplii ($F = 6.53$; $P = 0.014$). A slightly higher lipid content was observed in Art + PHB_a treatment (9.3 ± 0.7 % on DW) but this was not significantly different from Art + PHB_c treatment (8.3 ± 0.5 % on DW) ($F = 1.220$; $P = 0.302$). The whole-body contents of the two essential PUFAs LOA ($F = 8.514$; $P = 0.006$) and LNA ($F = 9.282$; $P = 0.004$) expressed on DW were significantly increased in Art + PHB_a treatment as compared to the control *Artemia* while these levels were not increased in Art + PHB_c treatment. The whole-body content of the n-3 essential HUFA EPA did not change although content expressed on total lipid significantly decreased in PHB-supplied treatments as compared to the control treatment ($F = 6.610$; $P = 0.013$). The n-3 essential HUFA DHA was not detected in any of the treatments when expressed on whole body and on total lipid. The total n-6 and n-3 essential FAs and their ratio on whole body significantly increased in Art + PHB_a treatment but these did not change in Art + PHB_c treatment.

Table 3.1 Effects of supplying dietary PHB either in crystalline or amorphous form on the total lipid contents (% on DW) and the contents of four essential FAs in *Artemia* expressed either on dry weight (mg g⁻¹ DW) or on total lipid (mg g⁻¹ lipid; values between parentheses). Values are means \pm SEM. Different superscript letters in a column indicate significant differences ($P \leq 0.05$; P -values in **bold**).

Treatments	Total lipid	Essential FAs				Total n-6 essential FAs	Total n-3 essential FAs	n-3/n-6 ratio
		<i>LOA</i> (18:2 (n-6))	<i>LNA</i> (18:3 (n-3))	<i>EPA</i> (20:5 (n-3))	<i>DHA</i> (22:6 (n-3))			
Starved (control)	6.5 \pm 0.2 ^b	3.2 \pm 0.2 ^b (48.8 \pm 4.1 ^a)	15.4 \pm 1.2 ^b (239.3 \pm 24.2 ^a)	1.7 \pm 0.1 ^a (26.9 \pm 2.0 ^a)	Not detected (Not detected)	3.2 \pm 0.2 ^b (48.8 \pm 4.1 ^a)	17.1 \pm 1.3 ^b (266.3 \pm 26.0 ^a)	5.5 \pm 0.1 ^b (5.4 \pm 0.1 ^b)
Art + PHB _c	8.3 \pm 0.5 ^a	3.2 \pm 0.1 ^b (38.9 \pm 1.8 ^a)	15.6 \pm 0.7 ^b (188.3 \pm 5.6 ^a)	1.7 \pm 0.0 ^a (20.7 \pm 1.4 ^b)	Not detected (Not detected)	3.2 \pm 0.1 ^b (38.9 \pm 1.8 ^a)	17.3 \pm 0.8 ^b (209.0 \pm 6.7 ^a)	5.4 \pm 0.1 ^b (5.4 \pm 0.1 ^b)
Art + PHB _a	9.3 \pm 0.7 ^a	3.9 \pm 0.1 ^a (43.2 \pm 4.2 ^a)	20.4 \pm 0.9 ^a (226.0 \pm 20.8 ^a)	1.7 \pm 0.1 ^a (18.6 \pm 1.6 ^b)	Not detected (Not detected)	3.9 \pm 0.1 ^a (43.2 \pm 4.2 ^a)	22.1 \pm 0.9 ^a (244.6 \pm 22.3 ^a)	5.7 \pm 0.1 ^a (5.7 \pm 0.1 ^a)
<i>P</i>-value	0.014	0.006 (0.188)	0.004 (0.157)	0.882 (0.013)		0.006 (0.188)	0.006 (0.156)	0.017 (0.034)

3.3.2 Experiment 2. PHB supplementation for *Artemia* nauplii: co-supplying PHB with the bacterial PHB degrader *C. testosteroni*

Table 3.2 shows the effects of co-supplying PHB with the bacterial PHB degrader *C. testosteroni* on the lipid contents and the contents of four essential FAs of *Artemia* nauplii. Co-supplying dietary PHB in crystalline form with *C. testosteroni* (Art + PHB_c + C_t) significantly increased the whole-body lipid content of the *Artemia* nauplii as compared to either the control *Artemia* or *Artemia* supplied with *C. testosteroni* only (Art + C_t). This value, however, was not significantly different from *Artemia* supplied of PHB in amorphous form with *C. testosteroni* (Art + PHB_a + C_t). The whole-body contents of the two essential PUFAs LOA ($F = 3.310$; $P = 0.049$) and LNA ($F = 4.284$; $P = 0.023$) significantly increased in treatments consisting either Art + PHB_c + C_t or Art + PHB_a + C_t as compared to either the control or Art + C_t treatment. The whole-body content of the n-3 essential HUFA EPA did not significantly change although its content on total lipid was significantly decreased in Art + PHB_c + C_t treatment ($F = 4.675$; $P = 0.017$). A slight increase in the EPA content was observed in Art + C_t treatment, however, this was not significantly different from the control treatment. The n-3 essential HUFA DHA was not detected in any of the treatments. The total n-6 and n-3 essential FAs on whole body significantly increased in the treatments consisting either Art + PHB_c + C_t or Art + PHB_a + C_t as compared to the control treatment, however, these were not significantly different in the treatment Art + C_t.

Table 3.2 Effects of co-supplying PHB in crystalline or amorphous form with the bacterial PHB degrader *C. testosteroni* on the total lipid contents (% on DW) and the contents of four essential FAs in *Artemia* expressed either on dry weight (mg g⁻¹ DW) or on total lipid (mg g⁻¹ lipid; in parentheses). Values are means \pm SEM. Different superscript letters in a column indicate significant differences ($P \leq 0.05$; P -values in **bold**).

Treatments	Total lipid	Essential FAs				Total n-6 essential FAs	Total n-3 essential FAs	n-3/n-6 ratio
		<i>LOA</i> (18:2 (n-6))	<i>LNA</i> (18:3 (n-3))	<i>EPA</i> (20:5 (n-3))	<i>DHA</i> (22:6 (n-3))			
Starved (control)	9.9 \pm 0.5 ^b	4.4 \pm 0.2 ^b (44.7 \pm 0.7 ^a)	23.1 \pm 1.1 ^b (234.9 \pm 4.4 ^a)	1.5 \pm 0.0 ^a (15.5 \pm 0.5 ^{ab})	Not detected (Not detected)	4.4 \pm 0.2 ^b (44.7 \pm 0.7 ^a)	24.7 \pm 1.1 ^b (250.4 \pm 4.4 ^a)	5.6 \pm 0.0 ^b (5.6 \pm 0.0 ^b)
Art + PHB _c + C _t	12.1 \pm 0.6 ^a	5.1 \pm 0.3 ^a (42.6 \pm 2.8 ^a)	27.5 \pm 1.7 ^a (227.7 \pm 14.9 ^a)	1.6 \pm 0.1 ^a (13.7 \pm 0.9 ^c)	Not detected (Not detected)	5.1 \pm 0.3 ^a (42.6 \pm 2.8 ^a)	29.1 \pm 1.8 ^a (241.4 \pm 15.7 ^a)	5.7 \pm 0.0 ^{ab} (5.7 \pm 0.0 ^{ab})
Art + PHB _a + C _t	11.0 \pm 0.4 ^{ab}	5.1 \pm 0.1 ^a (46.4 \pm 1.0 ^a)	27.6 \pm 0.7 ^a (252.2 \pm 5.1 ^a)	1.5 \pm 0.0 ^a (13.9 \pm 0.4 ^{bc})	Not detected (Not detected)	5.1 \pm 0.1 ^a (46.4 \pm 1.0 ^a)	29.1 \pm 0.7 ^a (266.2 \pm 5.4 ^a)	5.7 \pm 0.0 ^a (5.8 \pm 0.0 ^a)
Art + C _t	10.4 \pm 0.1 ^b	4.6 \pm 0.1 ^b (44.2 \pm 0.8 ^a)	24.0 \pm 0.6 ^b (230.4 \pm 6.4 ^a)	1.7 \pm 0.0 ^a (16.1 \pm 0.2 ^a)	Not detected (Not detected)	4.6 \pm 0.1 ^{ab} (44.2 \pm 0.8 ^a)	25.7 \pm 0.6 ^{ab} (246.5 \pm 6.5 ^a)	5.6 \pm 0.1 ^b (5.6 \pm 0.0 ^b)
<i>P</i>-value	0.009	0.049 (0.448)	0.023 (0.251)	0.120 (0.017)		0.049 (0.448)	0.030 (0.312)	0.027 (0.025)

3.3.3 Experiment 3. PHB supplementation for *Artemia*: co-supplying PHB to a HUFA-rich lipid emulsion

Table 3.3 shows the effects of co-supplying dietary PHB in crystalline form to a HUFA-rich lipid emulsion on the lipid contents and the contents of four essential FAs of *Artemia* nauplii. Results from the 2 x 2 factorial design showed a non-significant interaction between PHB and lipid treatments on the whole-body lipid contents of the *Artemia* nauplii ($F = 2.949$; $P = 0.124$). Lipid ($F = 47.536$; $P = 0.000$) but not PHB treatment ($F = 0.408$; $P = 0.541$) significantly increased this parameter with a mean total lipid content of 25.1 ± 1.9 % on DW in lipid-enriched *Artemia* while this was 12.3 ± 0.5 % on DW for non-lipid enriched *Artemia*. The interaction between PHB and lipid treatments on four essential FAs and their ratio was also not significant. Lipid but not PHB treatment significantly increased the two essential PUFAs LOA and LNA on whole-body and on total lipid. Similarly, content of n-3 essential HUFA EPA on whole-body and on total lipid was significantly increased in lipid treatment but not in PHB treatment. The n-3 essential HUFA DHA was detected in the treatments consisting either Art -PHB_c + L or Art + PHB_c + L. Lipid but not PHB treatment significantly increased the total n-6 and n-3 essential FAs on whole-body and on total lipid. PHB treatment, however, significantly decreased the content of total n-3 essential FAs on total lipid ($F = 5.355$; $P = 0.049$). In the case of non-PHB supplied treatments, significantly higher total n-3 essential FAs was seen in Art - PHB_c + L treatment as compared to the control treatment ($P = 0.044$). The ratio of the n-3/n-6 essential FAs on whole-body and total lipid significantly decreased in lipid treatment but did not change in PHB treatment.

Table 3.3 Effects of co-supplying PHB in crystalline form to HUFA-rich lipid emulsion on the total lipid contents (% on DW) and the contents of four essential FAs in *Artemia* expressed either on dry weight (mg g⁻¹ DW) or on total lipid (mg g⁻¹ lipid; in parentheses). Values are means \pm SEM. *P*-values in **bold** indicate significant differences ($P \leq 0.05$).

Treatments	Total lipid	Essential FAs				Total n-6 essential FAs	Total n-3 essential FAs	n3/n6 ratio
		<i>LOA</i> (18:2 (n-6))	<i>LNA</i> (18:3 (n-3))	<i>EPA</i> (20:5 (n-3))	<i>DHA</i> (22:6 (n-3))			
Starved (control)	11.3 \pm 0.4	4.9 \pm 0.2 (43.7 \pm 0.5)	26.2 \pm 0.8 (232.0 \pm 2.4)	1.6 \pm 0.0 (14.5 \pm 0.2)	Not detected (Not detected)	4.9 \pm 0.2 (43.7 \pm 0.5)	27.9 \pm 0.9 (246.6 \pm 2.4)	5.6 \pm 0.0 (5.6 \pm 0.0)
Art - PHB _c + L	27.3 \pm 2.7	17.2 \pm 1.9 (62.9 \pm 1.9)	39.7 \pm 0.6 (148.4 \pm 14.2)	16.7 \pm 2.6 (60.6 \pm 4.5)	19.9 \pm 4.2 (71.4 \pm 9.7)	17.2 \pm 1.9 (62.9 \pm 1.8)	76.4 \pm 7.3 (280.4 \pm 7.2)	4.5 \pm 0.1 (4.5 \pm 0.1)
Art + PHB _c - L	13.3 \pm 0.1	4.8 \pm 0.2 (36.0 \pm 2.0)	25.3 \pm 1.6 (190.0 \pm 13.0)	1.7 \pm 0.0 (12.5 \pm 0.3)	Not detected (Not detected)	4.8 \pm 0.2 (36.0 \pm 2.0)	26.9 \pm 1.6 (202.5 \pm 13.2)	5.6 \pm 0.1 (5.6 \pm 0.1)
Art + PHB _c + L	22.9 \pm 2.5	13.7 \pm 2.2 (58.9 \pm 4.6)	33.3 \pm 3.8 (145.4 \pm 6.5)	14.6 \pm 2.6 (62.8 \pm 4.9)	16.3 \pm 2.8 (70.0 \pm 5.3)	13.7 \pm 2.2 (58.9 \pm 4.6)	64.2 \pm 9.0 (278.1 \pm 13.0)	4.7 \pm 0.2 (4.7 \pm 0.2)
<i>P</i>-value								
PHB _c	0.541	0.240 (0.064)	0.121 (0.059)	0.586 (0.984)	0.512 (0.906)	0.240 (0.064)	0.298 (0.049)	0.200 (0.200)
L	0.000	0.000 (0.000)	0.001 (0.000)	0.000 (0.000)	* (*)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)
PHB _c x L	0.124	0.276 (0.516)	0.235 (0.094)	0.579 (0.543)	* (*)	0.276 (0.515)	0.368 (0.070)	0.121 (0.121)

* *P*-values for lipid (L) treatment and PHB_c x lipid interaction (PHB_c x L) cannot be determined since contents in treatments consisting either control or crystalline PHB-supplied *Artemia* were not detected.

3.4 Discussion

This study demonstrated the effects of supplying dietary PHB on the lipid contents and the contents of four essential FAs of *Artemia* nauplii. In all experiments, it was found that supplying dietary PHB resulted in increasing whole-body lipid contents of the starved *Artemia* nauplii indicating the lipid-saving effects of PHB.

PHB in amorphous form but not in crystalline form seemed to influence the levels of two essential PUFAs LOA and LNA as seen by the significantly higher contents of these FAs on whole-body. This difference might be due to the fact that PHB particles added in a bacterial carrier are smaller than crystalline PHB. For instance, Beeby *et al.* (2012) detected PHB granules of < 100 nm diameter in wild-type *R. eutropha* H16 with the smallest granule size being ~ 25 nm in diameter, while crystalline PHB used in the present study had an average size of 30 µm (De Schryver *et al.* 2010a). Neither of the two PHB forms affected the PUFA composition as similar values ($P > 0.05$) were observed among treatments, when PUFAs are expressed on total lipid contents. From this, it can be suggested that there is no preferential degradation of PUFAs in the presence of PHB.

Dietary PHB did not seem to influence the level of n-3 essential HUFA EPA on whole-body, however, it influenced the composition of n-3 essential HUFAs by decreasing the level of EPA when this FA is expressed on total lipid contents. Dietary PHB did not contribute to the increase in the content of n-3 essential HUFA DHA of the naturally DHA-deprived *Artemia* nauplii, however, its provision in amorphous form resulted in an increasing n-3/n-6 ratio. In juvenile narrow clawed crayfish *Astacus leptodactylus*, an

increase in n-3/n-6 ratio from 0.08 to 4.41 led to a significant increase in the weight gain (18 vs. 25 %), survival (59 vs. 69 %) and specific growth rate (0.26 vs 0.35 % day⁻¹) and reduced feed conversion ratio (FCR) (4.7 vs. 3.2) (Valipour *et al.* 2011). Guary *et al.* (1976) reported that diets containing higher levels of n-3 PUFA than n-6 PUFA in juvenile *P. japonicus* resulted in higher weight (33.9 vs. 32.4 %) and length (12.0 vs. 8.8 %). It was also demonstrated by Fenucci *et al.* (1981) that higher ratio of n-3 to n-6 FAs gives the best growth response for juvenile *P. stylirostris*. The increasing n-3/n-6 ratio seen for amorphous PHB-supplied *Artemia* might be favourable since n-3 FAs are needed for biosynthesis of longer chain PUFAs for tissue incorporation, whereas, the n-6 FAs are mainly utilized as energy sources (Sandifer & Joseph 1976).

The addition of the bacterial PHB degrader *C. testosteroni* was earlier shown to increase the protective effects of PHB (Thai *et al.* 2015; Halet *et al.* 2007), and it was therefore examined what could be the effect of co-supplying dietary PHB in crystalline or amorphous form with *C. testosteroni*. In the present study, it was found that this strategy also significantly improved the whole-body lipid contents of starved *Artemia* nauplii. In addition, the comparable levels of essential FAs and its ratio in treatment co-supplied crystalline PHB with *C. testosteroni* and treatment co-supplied amorphous PHB with *C. testosteroni* seemed to indicate that the addition on *C. testosteroni* could have effectively acted on the crystalline PHB and this may have resulted in better degradation of PHB into β -hydroxybutyrate (β -HB). Reports in literature have mentioned that *C. testosteroni* produce extracellular PHB depolymerase that could hydrolyze PHB into β -HB monomers (Mukai *et al.* 1993; Kasuya *et al.* 1994; Shinomiya *et al.* 1997). Results from a challenge

trial conducted by Defoirdt *et al.* (2007b) have shown that survival of PHB-supplemented *Artemia* challenged with *Vibrio campbelli* LMG21363 was enhanced with the addition of *C. testosteroni* at 10^7 CFU mL⁻¹. The PUFA composition, when expressed on total lipid, was not significantly different among treatments indicating the non-preferential degradation of PUFAs as mentioned previously.

Our earlier findings in Chapter 2 using postlarval tiger shrimp have shown that co-supplying dietary PHB in crystalline form with HUFA-rich lipid emulsion in a short-term (6-h) enrichment strategy nullified the beneficial effects of dietary PHB in terms of improving the survival of the postlarvae after exposure to either lethal dose of ammonium chloride or pathogenic *V. campbelli*. From that experiment, it was suggested that long-term enrichment (e.g. 24-h) of *Artemia* nauplii with HUFA-rich lipid emulsion and subsequent PHB treatment during the last hours of the enrichment process should be investigated whether this strategy could improve the nutritional contents of the *Artemia* and at the same time assure efficient delivery of PHB to the larval predator. In the present study, co-supplying PHB to lipid emulsion for 24 h did not seem to significantly affect the overall nutritional value of the lipid-supplied *Artemia* nauplii indicating that PHB may have not interfered with the beneficial effects of the HUFA-rich lipid emulsion.

In conclusion, the present study has demonstrated the lipid-saving effects of dietary PHB either supplied alone or in combination with the bacterial PHB degrader *C. testosteroni*. Supplying either only dietary PHB in crystalline or amorphous form or in combination with the bacterial PHB degrader *C. testosteroni* improved the essential FAs LOA and LNA on whole-body. PHB supplied alone or in combination with *C. testosteroni*

did not result in increasing content of the n-3 essential HUFA DHA. Finally, dietary PHB co-supplied to HUFA-rich lipid emulsion did not significantly affect the nutritional composition of the lipid-supplied *Artemia* nauplii.

Acknowledgement

The authors would like to thank Geert Vandewiele and Christ Mahieu of the Laboratory of Aquaculture and *Artemia* Reference Center (ARC) (Ghent University, Belgium) for the analysis of the samples and provision of *Artemia* sp. cysts, respectively.

Chapter 4

Assessment of poly- β -hydroxybutyrate (PHB) assimilation by crustaceans using stable ^{13}C isotope tracing

Chapter 4

ASSESSMENT OF POLY- β -HYDROXYBUTYRATE (PHB) ASSIMILATION BY CRUSTACEANS USING STABLE ^{13}C ISOTOPE TRACING

Gladys Ludevese-Pascual¹, Farhana Ahmed², Marleen De Troch², Edgar Amar³, Joseph Laranja^{1,3}, Samuel Bode⁴, Pascal Boeckx⁴, Peter Bossier^{1*} & Peter De Schryver^{1*}

¹ Laboratory of Aquaculture & *Artemia* Reference Centre, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University Rozier 44, B-9000 Gent, Belgium

² Marine Biology, Faculty of Sciences-Department of Biology, Ghent University Campus Sterre-S8, Krijgslaan 281, B-9000 Gent, Belgium

³ Southeast Asian Fisheries Development Centre/Aquaculture Department (SEAFDEC/AQD), Brgy. Buyuan, Tigbauan Iloilo 5021 Philippines

⁴ Isotope Bioscience Laboratory - ISOFYS, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

*Shared senior authorship

Abstract

The assimilation of ^{13}C -labeled *Ralstonia eutropha* DSM545 containing 75 % PHB on dry weight in two crustacean species, postlarval whiteleg shrimp *Litopenaeus vannamei* and *Artemia* sp. nauplii was studied. Supplementation of 2 % ^{13}C -labeled *R. eutropha*

DSM545 in the diet of postlarvae resulted in consistent enrichment of the two long-chain fatty acids (LCFAs; C14-C24) pentadecanoic acid (15:0) and palmitic acid (16:0) in the neutral, glyco- and phospholipid fractions indicating that the ^{13}C signal may not only originate from the PHB but partly from the structural components of the bacterial cell. There was a trend of lower ^{13}C enrichment in LCFAs from various lipid fractions of the postlarval whiteleg shrimp as compared to the diet indicating that PHB offered in natural matrix (e.g. contained in a bacterial carrier) may not be used as a building block but probably rather as energy source. The potential interference of diet composition on ^{13}C enrichment was also explored in the present study. The ^{13}C enrichment either in postlarval whiteleg shrimp fed low (6.2 % crude fat) or high fat (9.0 % crude fat) diet or in *Artemia* nauplii fed only ^{13}C -labeled *R. eutropha* DSM545 at 200 mg L⁻¹ or in combination with either *Tetraselmis suecica*, *Dunaliella tertiolecta* (both at 10⁵ cells mL⁻¹) or heat-killed cells of *Aeromonas hydrophila* (LVS3) at 10⁷ cells mL⁻¹ resulted in an overall non-significantly different $\delta^{13}\text{C}$ values indicating that assimilation of PHB offered in natural matrix is not influenced by diet composition. In the case of *Artemia*, the significantly low $\delta^{13}\text{C}$ in treatment Art + *T. suecica* + ^{13}C -labeled *R. eutropha* DSM545 as compared to Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545 may indicate that *T. suecica* is the preferred feed item over LVS3. Finally, there seemed to be a rapid transfer of ^{13}C in the tissues of postlarval whiteleg shrimp based on the higher $\delta^{13}\text{C}$ value at 4 h relative to 0 h irrespective of the diet. Similarly, significantly higher $\delta^{13}\text{C}$ levels were measured in *Artemia* nauplii after 2 h of incubation. These findings may indicate that PHB offered in natural matrix is rapidly assimilated in the tissues of crustaceans.

4.1 Introduction

The application of the bacterial storage compound poly- β -hydroxybutyrate (PHB) in aquatic farmed species has been reported to result in the promotion of growth (Nhan *et al.* 2010; Sui *et al.* 2012), improve robustness against adverse environmental conditions (Laranja *et al.* 2014) and enhance resistance against pathogens (Defoirdt *et al.* 2007b; Sui *et al.* 2012, Suguna *et al.* 2014, Halet *et al.* 2007, Laranja *et al.* 2014; Thai *et al.* 2014). PHB is also hypothesized to be degraded in the gut of the *Artemia* (Defoirdt *et al.* 2007b) where it functions as energy source (De Schryver *et al.* 2010a, Defoirdt *et al.* 2007a). In more recent findings, PHB has been shown to affect lipid metabolism in selected fish species (Najdegerami *et al.* 2015; Situmorang 2015). Despite the growing evidence on the beneficial effects of PHB in many aquatic farmed species, its assimilation in the tissues once ingested and degraded *in vivo* remained largely unexplored. Recent study employing the use of ^{13}C stable isotope analysis has demonstrated that PHB is assimilated and distributed in different organs of the Nile tilapia *Oreochromis niloticus* fingerlings (Situmorang 2015). However, there have been no reports described for crustaceans. The aim of the present study is to determine the assimilation of PHB offered in its natural matrix to two crustacean species, postlarval whiteleg shrimp *Litopenaeus vannamei* and *Artemia* sp. nauplii by means of ^{13}C stable isotope tracing. Considering the hypothesis that PHB and/or β -HB is used as energy source in aquatic farmed species, the assimilation of PHB in postlarval *L. vannamei* fed a basal diet containing either low- or high level of dietary fat is investigated to determine whether a low-fat diet could result in lower PHB assimilation or a high fat diet could result in higher PHB assimilation in the tissues of

crustaceans. Finally, the ^{13}C signal of different fatty acid groups in postlarval *L. vannamei* fed a diet supplemented with ^{13}C -labeled *R. eutropha* DSM545 is also examined to determine whether PHB is assimilated in the structural components of crustaceans.

4.2 Methodology

4.2.1 Axenic *Artemia* culture

Experiments 1, 2 and 3 were performed with *Artemia* sp. cysts, originating from Great Salt Lake, Utah (EG type; INVE Aquaculture NV, Belgium). Cysts were decapsulated according to the procedures described by Marques *et al.* (2005) and subsequently hatched via the procedure described in Chapter 3 (section 3.2). Axenic *Artemia* Instar II nauplii were subsequently treated according to the different treatments used in this study.

4.2.2 Whiteleg *L. vannamei* postlarvae

Experiment 4 and 5 were performed with postlarval whiteleg shrimp *L. vannamei* (0.11 ± 0.01 g). These postlarvae (PL10) were obtained from Shrimp Improvement Systems (Islamorada, Florida) and maintained on a recirculating system belonging to the Shrimp Facility of the Laboratory of Aquaculture and *Artemia* Reference Center (ARC), Ghent University Belgium. Salinity and temperature in the recirculating system averaged to 35 ppt and 28 °C, respectively. The photoperiod was maintained at 12 h light: 12 h dark cycle. During the rearing period, postlarvae were fed commercial diets (CreveTec bvba, Ternat, Belgium) containing 54 % crude protein and 12 % crude fat at a level of 10 % of

wet body weight (BW). The daily ration was divided into equal amounts and offered two times a day (9:00, 16:00 h).

4.2.3 ¹³C-labeled *Ralstonia eutropha* DSM545

The lyophilized cells of ¹³C-labeled *R.eutropha* DSM545 containing 75 % PHB on dry weight (DW) were produced by Vito NV, Belgium as described by Situmorang (2015). Before utilization, the lyophilized cells of *R. eutropha* were homogenized using a Polytron PT 10/35 homogenizer (Kinematica, Luzern Switzerland) for 30 sec.

4.2.4 *Aeromonas hydrophila* (LVS3)

A. hydrophila (LVS3) was grown overnight in Marine Broth (Difco Laboratories, Detroit, USA) at 28 °C on a shaker (150 rpm). Cells were harvested by centrifugation at 2 200 g for 15 mins at 4 °C and subsequently washed 3 times with filtered autoclaved seawater (FASW) and sterilized by autoclavation.

4.2.5 Algal cultures

The algal strains *Tetraselmis suecica* and *Dunaliella tertiolecta* obtained from the Culture Collection of Algae and Protozoa Department (CCAP) (Dunstaffnage Marine Laboratory, Scotland, UK) were maintained in ARC, Ghent University Belgium and grown using a standard Walne medium (Walne 1967) and FASW (Marques *et al.* 2004).

4.2.6 Experiment 1: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 by axenic *Artemia* nauplii

Axenic *Artemia* Instar II were distributed at a density of 200 nauplii mL^{-1} to sterile 12 mL cylindrical container with an outside diameter of 46 mm and a height of 35 mm containing 10 mL of FASW.

These nauplii were given the following dietary treatments ($n = 3$):

-LVS3 only (Art + LVS3)

-LVS3 + ^{13}C -labeled *R. eutropha* DSM545 (Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545)

Containers were placed on a rotor perpendicular to the axis (5 rpm) and incubated at 28 °C with constant illumination (fluorescence tubes) of $27 \mu\text{E m}^{-2}\text{s}^{-1}$ for 48 h. LVS3 cells were given to the treatments at a density of 10^7 cells mL^{-1} (approximately 10 mg L^{-1}) while ^{13}C -labeled *R. eutropha* DSM545 was supplemented at 200 mg L^{-1} .

After 48 h, the *Artemia* nauplii were recovered on a 200 μm sieve and washed thoroughly with FASW to remove residual diet. Next, the nauplii were axenically transferred to new 12 mL containers containing 10 mL of FASW and mounted back to the rotor for a 10 h starvation period to evacuate the gut. Finally, the nauplii were concentrated on a 200 μm sieve, transferred carefully to tin capsules (8 x 5 mm; Sercon, UK) and oven-dried at 60 °C for 12 h (Heraeus Hanau, Germany). Dried samples were analyzed for $\delta^{13}\text{C}$ (‰) using an elemental analyzer (EA) (ANCA-SL, SerCon, UK) coupled to an Isotope

Ratio Mass Spectrometer (IRMS) (20-20, SerCon, UK) at the Isotope Bioscience Laboratory of Ghent University (Ghent, Belgium).

*4.2.7 Experiment 2: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 by xenic *Artemia* nauplii as influenced by dietary composition*

Artemia Instar II nauplii obtained as described previously were distributed at a density of 200 nauplii mL^{-1} to 50 mL cylindroconical tubes containing 40 mL of FASW. These nauplii were given the following dietary treatments ($n = 3$):

-*T. suecica* only (Art + *T. suecica* ; D1)

- ^{13}C -labeled *R. eutropha* DSM545 only (Art + ^{13}C -labeled *R. eutropha* DSM545; D2)

-*T. suecica* + ^{13}C -labeled *R. eutropha* DSM545 (Art + *T. suecica* + ^{13}C -labeled *R. eutropha* DSM545; D3)

-LVS3 + ^{13}C -labeled *R. eutropha* DSM545 (Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545; D4)

-*D. tertiolecta* + ^{13}C -labeled *R. eutropha* DSM545 (Art + *D. tertiolecta* + ^{13}C -labeled *R. eutropha* DSM545; D5)

Tubes were placed on a rotor perpendicular to the axis (5 rpm) and incubated at 28 °C with constant illumination of $27 \mu\text{E m}^{-2}\text{s}^{-1}$ for 48 h. The algal species *T. suecica* and *D. tertiolecta* were supplied at a density of 10^5 cells mL^{-1} , approximately 14 mg L^{-1} and 3.4

mg L⁻¹, respectively, while ¹³C-labeled *R. eutropha* DSM545 and LVS3 were given as mentioned previously. After 48 h, the *Artemia* nauplii were recovered on a 200 µm sieve and subjected to a 10 h starvation period as described previously for Experiment 1.

4.2.8 Experiment 3: Assimilation of ¹³C-labeled *R. eutropha* DSM545 by xenic *Artemia* nauplii as influenced by time

Artemia Instar II obtained as described previously were distributed at 200 nauplii mL⁻¹ to 1 L sterile incubation bottles containing 500 mL FASW and equipped with two 0.20 µm filters (Sartorius) on the aeration in- and outlet. These nauplii were supplemented the following dietary treatments ($n = 3$):

-*T. suecica* only (Art + *T. suecica*)

-*T. suecica* + ¹³C-labeled *R. eutropha* DSM545 (Art + *T. suecica* + ¹³C-labeled *R. eutropha* DSM545)

Bottles were incubated at 28 °C with constant illumination of 27 µE m⁻²s⁻¹ for 96 h. ¹³C-labeled *R. eutropha* DSM545 was provided as described previously while xenic *T. suecica* were given on a daily basis at a density of 10⁵ cells mL⁻¹ (approximately 14 mg L⁻¹) (Marques *et al.* 2004). *Artemia* were sampled by collecting 50 mL from each bottle after vigorous shaking at different time points (0, 2, 4, 6, 12, 24, 48 and 96 h). For the samples collected at 0 h, only samples taken from the treatment Art + *T. suecica* were considered. These *Artemia* samples were concentrated on a 200 µm sieve, washed thoroughly with FASW to remove residual diet and resuspended in 50 mL cyindroconical tubes containing 40 mL of cellulose suspension at 1.5 g L⁻¹ to purge enrichment particles from the gut of the

nauplii (Niu *et al.* 2012). Tubes were placed back on a rotor (5 rpm) and incubated at 28 °C with illumination set at 27 $\mu\text{E m}^{-2}\text{s}^{-1}$ for 6 h. At the end of the cellulose purging, *Artemia* were collected on a 200 μm sieve, transferred to tin capsules (8 x 5 mm; Sercon, UK) and oven-dried as described before. Samples were analyzed for $\delta^{13}\text{C}$ using a PDZ Europe ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrophotometer at the UC Davis Stable Isotope Facility (Department of Plant Sciences, University of California, USA).

4.2.9 Experiment 4: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 in Whiteleg *L. vannamei* postlarvae as influenced by dietary composition

Based on the preliminary results obtained on the influence of different feed items on the assimilation of ^{13}C -labeled *R. eutropha* DSM545 in *Artemia* nauplii, the assimilation of ^{13}C -labeled *R. eutropha* DSM545 containing 75 % PHB on DW by postlarval *L. vannamei* was verified. Postlarvae were stocked at 1 individual L^{-1} in two acclimation tank consisting of 50 L plastic tank. Temperature and level of dissolved oxygen in the acclimation tanks were maintained at 28 °C and $\geq 5 \text{ mg L}^{-1}$, respectively. During this period, postlarvae were acclimated to a sub-optimal (6.2 % crude fat) or an optimal (9.0 % crude fat) diet containing 2 % non ^{13}C -labeled *R. eutropha* DSM545. Diets which were given at a level of 10 % on wet BW were divided into two feeding sessions daily (9:00 and 16:00 h). The composition of the different diets used during the acclimation period is shown in Table 4.1. The uneaten feed and faecal matter were removed daily using a siphon and approximately 50 % of the rearing water was replaced daily with pre-heated (28 °C) seawater in order to maintain levels of total ammonia-

nitrogen, nitrite-nitrogen and nitrate-nitrogen below 0.2, 0.1, and 10.0 mg L⁻¹, respectively. At the end of the 14-day acclimation period, postlarvae were transferred to the experimental set-up.

The experimental units consisted of 1500 mL rectangular plastic reservoir filled with 1 L pre-heated (28 °C) seawater with individual cover contained a single postlarva that was fed either sub- or optimal diet for a period of 7 days to acclimate them to the new environment. Diets were given as mentioned previously. Air was continuously provided in each unit through the cover. These units were placed in a larger tank containing seawater and provided with heater (100 W, JBL Neuhofen Germany) serving as a water bath. The temperature in the water bath system was maintained at 28 °C. Photoperiod was kept at 12 h light: 12 h dark cycle and water change was administered daily at 50 %. Next, the postlarvae were fed a single dose of either sub- or optimal diet (during the first feeding session) having the same diet composition as mentioned previously, but this time, containing 2 % ¹³C-labeled *R. eutropha* DSM545 instead of non ¹³C-labeled lyophilized cells of *R. eutropha* DSM545. Feeding with non-labeled diets resumed in the second feeding session. Postlarvae ($n = 4$ biological replicates) were sampled at 0, 4, 8, 16, 32, 48, 72 and 120 h. These postlarvae were degutted and transferred to 1.5 mL tubes, flash-frozen in liquid nitrogen and stored at – 80 °C until further analysis. Frozen samples of postlarvae were oven-dried at 60 °C until constant weight. Subsequently, dried samples of postlarvae were homogenized using a mortar and a pestle and 1 ± 0.1 mg of the homogenized sample was separated and carefully transferred to tin capsules (8 x 5 mm; Sercon, UK) and pinched closed for analysis of $\delta^{13}\text{C}$ (‰) as mentioned previously.

Table 4.1. Dietary feed composition.

Feed ingredients	Composition (% on DW)	
	Sub-optimal diet	Optimal diet
Soy flour	30.3	30.3
Wheat flour	28.2	28.2
Fish meal 82	13.2	13.2
Poultry by product meal	7.4	7.4
Sepiolite 5	5.0	2.3
Vitamins and minerals premix	3.9	3.9
Gelatine 3	2.9	2.9
Tuna oil	1.8	1.8
Calcium carbonate	1.6	1.6
Monosodium phosphate	1.3	1.3
Soybean oil	1.0	3.7
Lecitine powder 95	0.8	0.8
Salt	0.5	0.5
Cholesterol 80 % XG	0.2	0.2
PHB amorphous	2.0	2.0

4.2.10 Experiment 5: Fatty acid-specific stable isotope analysis

The postlarval whiteleg shrimp were subjected to a 14-day acclimation period as previously described. At the end of the acclimation period, postlarvae were transferred to the experimental set-up.

The experimental set-up and conditions applied in this experiment were similar to Experiment 4. This time, however, all postlarvae were fed only 1 type of diet (optimal diet) for a period of 7 days to acclimate them to the new environment. Next, postlarva was given a single dose (50 % of the daily feeding ration) of optimal diet containing 2 % ^{13}C -labeled *R. eutropha* DSM545 during the first feeding session and feeding with non-labeled diet

resumed in the second feeding session. The postlarvae ($n = 4$) were sampled at the start (0 h) and after 24 h. Sampling was done as described in Experiment 4.

4.2.11 Lipid extraction and fractionation

The freeze-dried shrimp samples were extracted using a modified Bligh-Dyer method (Rütters *et al.* 2002; Bligh & Dyer 1959). Briefly, 100 mg of homogenized whole shrimp sample was extracted with methanol-chloroform-water (3:1.5:2 vol/vol/vol) by shaking (INNOVA 2000) at 130 rpm for 2 h. Next, the supernatants were collected, and chloroform (1.5 mL) and MilliQ water (1.5 mL) were added to enhance phase separation. The chloroform fraction was transferred to a 10 mL round-bottom tube, dried under a nitrogen flow and stored dry at $-20\text{ }^{\circ}\text{C}$. Separation of total lipids into neutral lipids, glycolipids and phospholipids was achieved on a chloroform-prerinsed silica column (0.5 g; activated for 3 h at $150\text{ }^{\circ}\text{C}$) by eluting with 2.5 mL chloroform, 2.5 mL acetone, and 5 mL methanol, respectively (Boschker 2004). The resulting fractions were dried under nitrogen and stored at $-20\text{ }^{\circ}\text{C}$ for further analysis (Heinzelmann *et al.* 2014).

4.2.12 Fatty acid-specific stable isotope analysis

The isotopic composition of the prepared fatty acids methyl esters (FAMES) was determined using gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS; constituted of a Trace GC Ultra interfaced by GC/C III interface to Delta^{Plus}XP IRMS, all Thermo Scientific, Bremen Germany) equipped with a BPX5 column (30 m, 0.25 mm i.d., 0.5 μm film thickness; SGE, USA). The identity of individual FAME was

based on retention times and ion composition (mass spectrometry) compared to multiple reference materials (Bode, *pers. comm*; Deneff *et al.* 2009).

$\delta^{13}\text{C}$ analysis

All $\delta^{13}\text{C}$ values were defined in delta notation (δ) and reported in parts per thousand (‰ or per mil). Values were calculated following the formula (Middelburg *et al.* 2000):

$$\delta^{13}\text{C} = \left(\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right) \times 1000$$

where $\delta^{13}\text{C}$ is the stable isotope ratio while R_{sample} and R_{standard} are the molar ratio of the heavy/light isotope ($^{13}\text{C}/^{12}\text{C}$) of the sample and the reference, respectively. Samples were referenced against the international standard Vienna Pee Dee Belemnite (VPDB; 0.0112372). Routine measurements were precise to within ± 0.2 ‰ for natural samples while ± 0.4 ‰ for enriched materials (Situmorang 2015).

4.2.13 Statistical analysis

Descriptive statistics were used to assess normality of the data before and after various transformations. Data obtained from Experiment 1 were analyzed using the non-parametric Mann-Whitney *U*-test to compare treatment means. Data from Experiment 2 were analyzed with a one-way analysis of variance (ANOVA) followed by a Duncan's multiple range test using 95 % confidence limits while data in Experiment 3 were analyzed either with an Independent samples T-test or the non-parametric Mann-Whitney *U*-test to compare data to the control or time 0. Repeated measures ANOVA (RM-ANOVA) was used in Experiment 4 in which data were presented in a cumulative way to evaluate

changes in isotopic ratio at multiple time points. Data sphericity was tested prior the RM-ANOVA by means of the Mauchly's test to check for the assumption that there was no significant interaction among time points and diets. An Independent samples T-test was also used to analyze data in Experiment 5 to compare data to the control or time 0. All statistical analyses were performed using SPSS Statistical software v. 11.5 with alpha level set at 0.5. Graphic presentations of results were carried with Sigmaplot 13.0 (Systat Software, Inc., Chicago, IL, USA).

4.3 Results

4.3.1 Experiment 1: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 by axenic *Artemia nauplii*

The mean $\delta^{13}\text{C}$ in Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545 treatment was $2\ 341.4 \pm 100.0\ ‰$ and this was significantly higher as compared to Art + LVS3 treatment with mean $\delta^{13}\text{C}$ of $-18.4 \pm 0.2\ ‰$ ($U = 0$; $P = .05$).

4.3.2 Experiment 2: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 by xenic *Artemia nauplii* as influenced by dietary composition

Figure 4.1 shows the mean $\delta^{13}\text{C}$ of *Artemia* nauplii supplied with either *T. suecica*, *D. tertiolecta* or LVS3 combined with or without ^{13}C -labeled *R. eutropha* DSM545. Mean $\delta^{13}\text{C}$ in the control diet consisting of *T. suecica* alone was $-18.2 \pm 0.2\ ‰$ and this was significantly lowest as compared to any dietary treatment. The mean $\delta^{13}\text{C}$ in Art + ^{13}C -labeled *R. eutropha* DSM545 treatment was $4\ 075.8 \pm 328.9\ ‰$ and this was not significantly different in treatments consisting either Art + *T. suecica* + ^{13}C -labeled *R.*

eutropha DSM545, Art + LVS3 ^{13}C -labeled *R. eutropha* DSM545 or *D. tertiolecta* + ^{13}C -labeled *R. eutropha* DSM545 with mean $\delta^{13}\text{C}$ values of $3\,108.6 \pm 96.3$ ‰, $4\,639.8 \pm 828.9$ ‰ and $4\,382.1 \pm 363.5$ ‰, respectively. The treatment consisting of Art + *T. suecica* + ^{13}C -labeled *R. eutropha* DSM545 had significantly lower mean $\delta^{13}\text{C}$ as compared to treatment consisting of Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545 but this was not significantly different from the other treatments except for the control diet consisting of *T. suecica* alone.

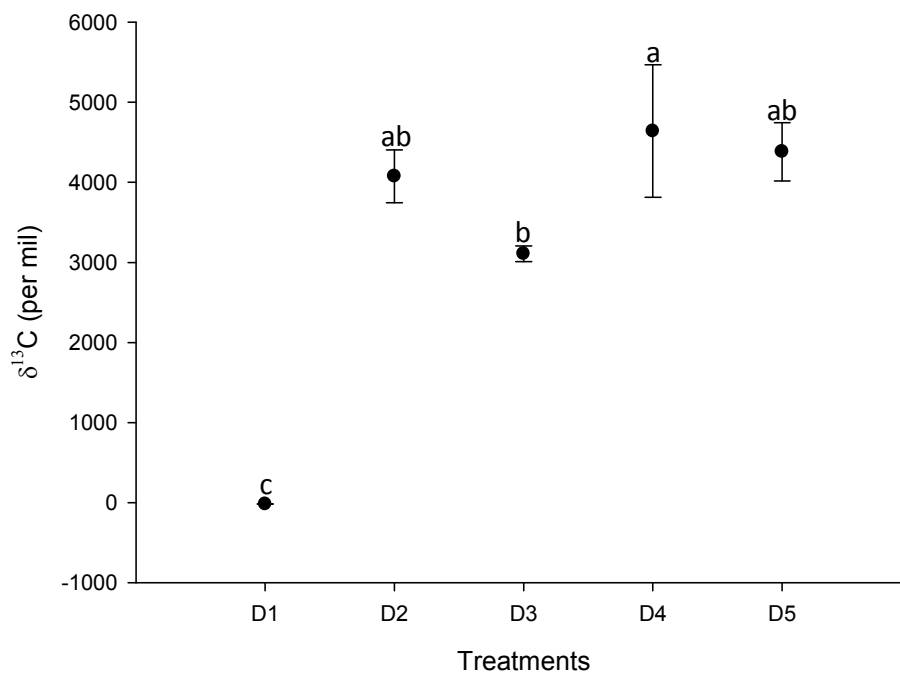


Figure 4.1 Mean $\delta^{13}\text{C}$ of *Artemia* nauplii supplied different dietary treatments (Art + *T. suecica* (D1); Art + ^{13}C -labeled *R. eutropha* DSM545 (D2); Art + *T. suecica* + ^{13}C -labeled *R. eutropha* DSM545 (D3); Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545 (D4); Art + *D. tertiolecta* + ^{13}C -labeled *R. eutropha* DSM545 (D5)) in

48 h. Values are presented as mean \pm SEM ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$).

4.3.3 Experiment 3: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 by xenic *Artemia* nauplii as influenced by time

Figure 4.2 shows the mean $\delta^{13}\text{C}$ of *Artemia* nauplii supplied a combination of *T. suecica* and ^{13}C -labeled *R. eutropha* DSM545. *Artemia* nauplii supplied *T. suecica* alone had a mean $\delta^{13}\text{C}$ value of -20.9 ± 0.3 ‰ and this significantly increased to 238.0 ± 10.2 ‰ in *Artemia* supplied a combination of *T. suecica* and ^{13}C -labeled *R. eutropha* DSM545 after 2 h.

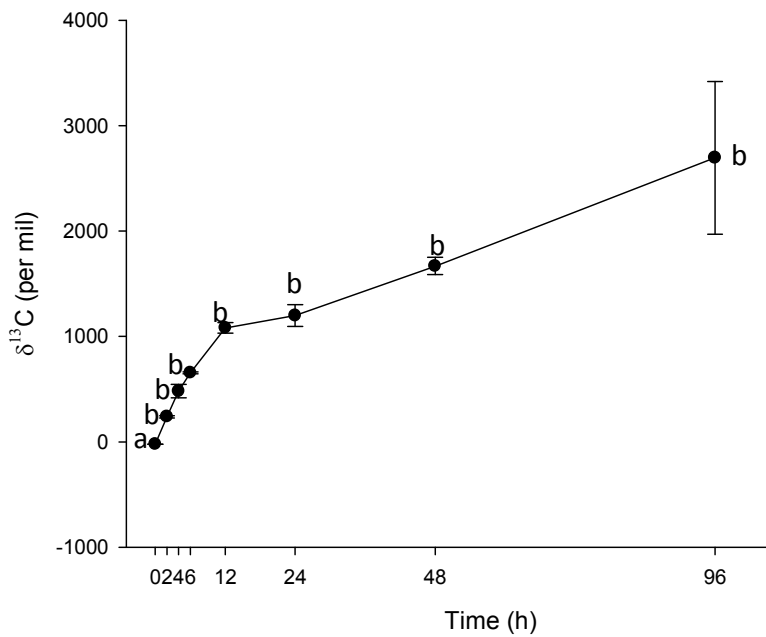


Figure 4.2 Mean $\delta^{13}\text{C}$ of *Artemia* nauplii in function of time. Values are presented as mean \pm SEM ($n = 3$). Different letters indicate significant differences from time 0 ($P \leq 0.05$).

At 4 h, the $\delta^{13}\text{C}$ value increased to $481.1 \pm 62.8 \text{ ‰}$ but this was not significantly different from time point 2 h. A trend of increasing mean $\delta^{13}\text{C}$ was seen until time point 96 h ($695.1 \pm 723.2 \text{ ‰}$) and these values were significantly higher as compared to time 0.

4.3.4 Experiment 4: Assimilation of ^{13}C -labeled *R. eutropha* DSM545 in Whiteleg *L. vannamei* postlarvae as influenced by dietary composition

Figure 4.3 shows the mean $\delta^{13}\text{C}$ of postlarval whiteleg shrimp fed ^{13}C -labeled *R. eutropha* DSM545 supplemented either in sub- or optimal diet. Results from the 8 x 2 factorial design with repeated measures showed a non-significant interaction between time and diet ($F(7, 28) = 1.56, P = 0.184$). The main effect of time treatment ($F(7, 28) = 4.82, P = 0.001$) was significant but this was not the case for the diet treatment ($F(1, 4) = 0.55, P = 0.500$). Pairwise comparison between sub- and optimal diets at time point 16 h showed significantly higher mean $\delta^{13}\text{C}$ in postlarvae fed ^{13}C -labeled *R. eutropha* DSM545 supplemented in sub-optimal diet ($17.9 \pm 6.1 \text{ ‰}$) as compared to postlarvae fed ^{13}C -labeled *R. eutropha* DSM545 supplemented in optimal diet ($3.7 \pm 4.3 \text{ ‰}$) but this did not differ at other time points.

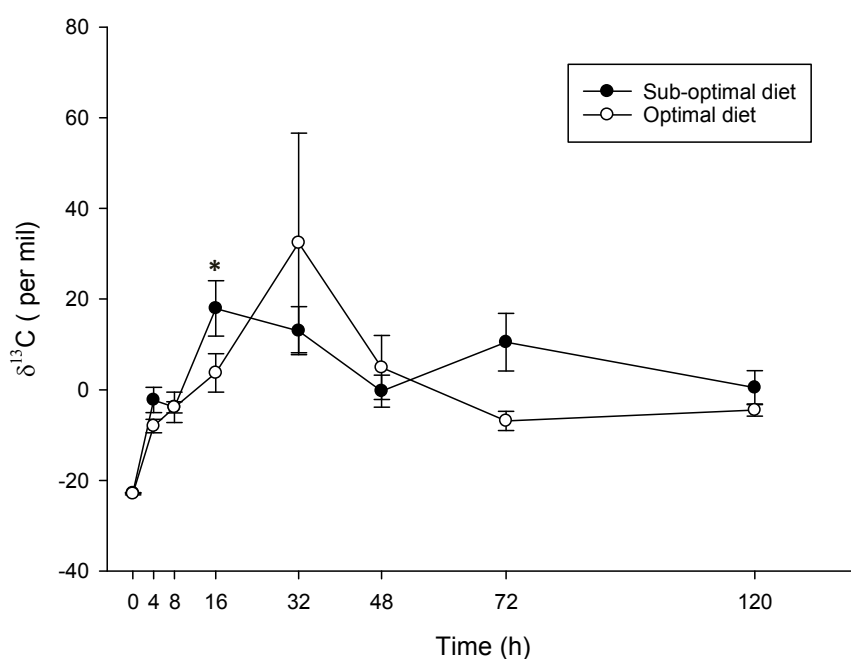


Figure 4.3 Mean $\delta^{13}\text{C}$ of postlarval whiteleg shrimp fed ^{13}C -labeled *R. eutropha* DSM545 supplemented either in sub- or optimal diets. Values represent mean \pm SEM ($n = 4$). Asterisk indicates significant differences between dietary treatments within a time point ($P \leq 0.05$).

4.3.5 Experiment 5: Fatty acid-specific stable isotope analysis

Table 4.2 shows the $\delta^{13}\text{C}$ values with their corresponding contents expressed as area % in diet supplemented either non-labeled *R. eutropha* DSM545 or ^{13}C -labeled *R. eutropha* DSM545. The area % of the LCFAs in the two diets closely resembled each other. There was a major ^{13}C enrichment in most LCFAs belonging to the saturated and monounsaturated FA groups in diet supplemented with 2 % ^{13}C -labeled *R. eutropha* DSM545. Further, it also resulted in ^{13}C enrichment of two LCFAs considered essential to

penaeid shrimp: linoleic acid (LOA; 18:2(n-6)) and eicosapentanoic acid (EPA; 20:5(n-3)). The two other LCFAs considered essential to penaeid shrimp, namely docosahexaenoic acid (DHA; 22:6(n-3)) and linolenic acid (LNA; 18:3(n-3)) were either non-enriched with ^{13}C or not detected in both diets.

The $\delta^{13}\text{C}$ values of the LCFAs in the neutral, glycolipid and phospholipid fractions of the postlarval whiteleg shrimp after 24 h are shown in Table 4.3, 4.4 and 4.5, respectively. Table 4.6 presents the LCFAs in various lipid fractions that were significantly enriched and their corresponding contents expressed in area %. The saturated LCFA palmitic acid (16:0) was significantly enriched in both neutral and glycolipid fractions. Although this LCFA was also enriched in the phospholipid fraction, no statistical support was made due to insufficient number of replicates. The saturated LCFA stearic acid (18:0) in glycolipid and phospholipid fractions were significantly enriched. The monounsaturated LCFAs, namely: palmitoleic acid (16:1(n-7)), heptadecenoic acid (17:1(n-7)), oleic acid (18:1(n-9)), vaccenic acid (18:1(n-7)) and erucic acid (22:1(n-9)) were significantly enriched in one or two lipid fractions. Significant enrichment in one or two lipid fractions was also observed for the n-6 LCFAs arachidonic acid (20:4(n-6)) and docosadienoic acid (22:2(n-6)). The n-3 LCFA eicosatrienoate (20:3(n-3)) was significantly enriched in the phospholipid fraction only.

Table 4.2 $\delta^{13}\text{C}$ values and their corresponding contents (expressed in area %) in diet either supplemented with non-labeled *R. eutropha* DSM545 or ^{13}C -labeled *R. eutropha* DSM545. Values are from two replicates (separated by semi-colon).

Long-chain fatty acid groups (LCFAs)	Carbon:Double bond (C:D)	Non ¹³ C-labeled <i>R. eutropha</i> supplemented diet		¹³ C-labeled <i>R. eutropha</i> DSM545 supplemented diet	
		Area %	δ ¹³ C	Area %	δ ¹³ C
Saturated LCFAs					
Myristic acid	14:0	1.22; 1.19	-23.18; -23.24	1.24; 1.19	428.37; 422.11
Pentadecanoic acid	15:0	0.21; 0.22	-14.22; -3.60	0.23; 0.22	2 473.53; 2 143.55
Palmitic acid	16:0	15.61; 15.69	-26.95; -27.50	15.87; 15.70	308.55; 291.74
Heptadecanoic acid	17:0	0.32; 0.32	-27.98; -28.10	0.34; 0.33	338.17; 328.18
Stearic acid	18:0	5.03; 5.03	-28.51; -29.32	5.09; 5.10	-6.26; -7.74
Nonadecylic acid	19:0	0.06; 0.07		0.05; 0.07	
Arachidic acid	20:0		-21.78; -23.73	0.03*	-210.19; -149.20
Docosanoic acid	22:0	0.25; 0.26	-35.65*	0.25; 0.27	-32.90; -32.04
Tetracosanoic acid	24:0		-32.00; -37.21		-65.80; -56.11
Monounsaturated LCFAs					
Myristoleic acid	14:1(n-5)	0.09; 0.08	-16.62*	0.09; 0.08	5 414.96; 5 644.25
Pentadecenoic acid	15:1(n-5)	0.03; 0.03		0.03*	97 283.60; 96 017.66
Palmitoleic acid	16:1(n-7)	1.85; 1.83	-23.88; -24.11	1.81; 1.81	506.76; 496.30
Heptadecenoic acid	17:1(n-7)	0.39; 0.38	17.51; 6.17	0.38; 0.38	29 626.50*
Oleic acid	18:1(n-9)	19.04; 19.12	-28.44; -29.55	19.04; 19.10	-22.08; -20.11
Vaccenic acid	18:1(n-7)	1.94; 1.96	-26.88; -33.82	1.92; 1.90	204.28; 218.69
	19:1(n-9)	0.08; 0.07		0.09; 0.08	
Eicosenoic acid	20:1(n-9)	0.88; 0.88		0.86; 0.84	
Paullinic acid	20:1(n-7)	0.11*		0.12; 0.10	

Erucic acid	22:1(n-9)	0.13;0.14		0.11; 0.13	
	22:1(n-7)	0.03;0.03		0.03*	
Nervonic acid	24:1(n-9)	0.15;0.17	-28.93; -29.54	0.12; 0.20	142.87; 165.96
n-6 LCFAs					
	18:2(n-6)-t	0.07; 0.07		0.07; 0.07	
Linoleic acid	18:2(n-6)-c	34.84; 35.18	-29.78; -31.92	34.94; 34.76	-20.00; -21.69
Gamma-linolenic acid	18:3(n-6)	0.04*		0.06; 0.04	
Dihomo-gamma-linolenic acid	20:3(n-6)	0.07;0.07	-42.21*	0.07; 0.07	32.17; 36.56
Arachidonic acid	20:4(n-6)	0.71;0.71	-29.77; -26.79	0.72; 0.72	1 593.22; 1 814.19
Ecosadienoic acid	20:2(n-6)		-30.72; -31.71		-33.50;34.10
Adrenic acid	22:4(n-6)	0.11;0.12		0.12; 0.12	
Docosapentaenoic acid					
(Osbond acid)	22:5(n-6)	0.42; 0.42		0.44; 0.44	
Docosadienoic acid	22:2(n-6)		-32.21; -35.34		-34.87*
n-3 LCFAs					
Alpha-linolenic acid	18:3(n-3)	5.27; 5.33		5.28; 5.26	
Stearidonic acid	18:4(n-3)	0.50; 0.49		0.52; 0.51	
Eicosatrienoate	20:3(n-3)	0.07; 0.07		0.07; 0.07	
Eicosatetraenoic acid	20:4(n-3)	0.15; 0.16		0.16; 0.16	
Eicosapentenoic acid	20:5(n-3)	2.20; 2.21	-31.02; -31.40	2.17; 2.18	33.66; 33.39
Heneicosapentaenoic acid	21:5(n-3)	0.06; 0.06		0.06*	
Docosapentaenoic acid					
(Clupanodonic acid)	22:5(n-3)	0.50; 0.50		0.46; 0.51	
Docosahexaenoic acid	22:6(n-3)	5.85; 5.89	-28.95; -29.93	6.05; 6.08	-31.60; -31.92

*Only 1 replicate

Empty cells = no values obtained

Table 4.3 Mean $\delta^{13}\text{C}$ values ($\text{‰} \pm \text{SEM}$) for fraction of neutral lipid extracted from degutted postlarval whiteleg shrimp ($n = 3$ or 4). Different letter superscript indicates significant differences ($P \leq 0.05$).

Long-chain fatty acid groups (LCFAs)	Carbon:Double bond (C:D)	0 h	24 h
Saturated LCFAs			
Lauric acid	C12:0	-19.33; -20.05**	
Pentadecanoic acid	C15:0	-24.91*	6.44*
Palmitic acid	C16:0	-24.64 \pm 0.46^a	16.32 \pm 1.73^b
Heptadecanoic	C17:0	-25.62*	-18.14 \pm 2.53
Stearic acid	C18:0	-25.02 \pm 0.51 ^a	-23.21 \pm 1.99 ^a
Tricosylic acid	C23:0	-32.39 *	
Hexacosanoic acid	C26:0	-24.14 \pm 0.65 ^a	-24.86 \pm 0.45 ^a
Monounsaturated LCFAs			
Pentadecenoic acid	C15:1(n-5)		-18.29*
Palmitoleic acid	C16:1(n-7)	-22.35*	80.96; 102.31**
Heptadecenoic acid	C17:1(n-7)	-25.40*	38.46 \pm 3.30
Oleic acid	C18:1(n-9)	-25.42 \pm 0.59^a	-22.01 \pm 0.68^b
Vaccenic acid	C18:1(n-7)	-25.37 \pm 0.48^a	53.34 \pm 7.90^b
Eicosenoic acid	C20:1(n-9)	-24.40*	-20.17*
n-6 LCFAs			
Linoleic acid	C18:2(n-6)	-26.90 \pm 0.53 ^a	-27.70 \pm 0.91 ^a
Arachidonic acid	C20:4(n-6)	-25.64 \pm 0.24^a	-21.71 \pm 1.77^b
Ecosadienoic acid	C20:2(n-6)	-26.04; -28.64**	-25.77 \pm 2.06
n-3 LCFAs			
Docosaheptaenoic	C22:6(n-3)	-21.75 \pm 0.70 ^a	-23.41 \pm 0.43 ^a
Eicosapentenoic acid	C20:5(n-3)	-22.81 \pm 0.63 ^a	-23.64 \pm 0.54 ^a

*Only 1 replicate

**Only 2 replicates (separated by semi-colon)

Table 4.4. Mean $\delta^{13}\text{C}$ values ($\text{‰} \pm \text{SEM}$) for fraction of glycolipids extracted from degutted postlarval whiteleg shrimp ($n = 3$ or 4). Different letter superscript indicates significant differences ($P \leq 0.05$).

Long-chain fatty acid groups (LCFAs)	Carbon:Double bond (C:D)	0 hr	24 h
Saturated LCFAs			
Lauric acid	C12:0	-20.33; -19.43**	
Myristic acid	C14:0	-22.45*	-8.31; -6.52**
Pentadecanoic acid	C15:0	-23.49*	16.21; 36.88**
Palmitic acid	C16:0	-25.22 \pm 0.28^a	21.78 \pm 1.15^b
Heptadecanoic acid	C17:0	-26.06; -26.03**	-11.02 \pm 5.15
Stearic acid	C18:0	-25.73 \pm 0.45^a	-15.95 \pm 3.04^b
Arachidic acid	C20:0	-26.70; -25.74**	-24.95 \pm 3.38
Tricosylic acid	C23:0	-27.06; -28.91**	-25.45 \pm 0.19
Lignoceric acid	C24:0	-26.15; -27.20**	
Hexacosanoic acid	C26:0	-24.06 \pm 0.58 ^a	-25.07 \pm 0.43 ^a
Monosaturated LCFAs			
Pentadecenoic acid	C15:1(n-5)	-24.13*	17.90 \pm 19.47
Palmitoleic acid	C16:1(n-7)	-22.66 \pm 0.19^a	83.32 \pm 8.58^b
Heptadecenoic acid	C17:1(n-7)	-23.89*	243.70; 175.73**
Oleic acid	C18:1(n-9)	-26.27 \pm 0.51^a	-20.91 \pm 0.80^b
Vaccenic acid	C18:1(n-7)	-24.61 \pm 0.62^a	38.13 \pm 4.38^b
Eicosenoic acid	C20:1(n-9)	-23.73 \pm 0.69 ^a	-18.96 \pm 2.87 ^a
Erucic acid	C22:1(n-9)	-27.12 \pm 0.17^a	-22.99 \pm 0.19^b
n-6 LCFAs			
Arachidonic acid	C20:4(n-6)	-26.02 \pm 0.11 ^a	-18.91 \pm 4.32 ^a
Dihomo-gamma-linolenic acid	C20:3(n-6)		-16.28*
Ecosadienoic acid	C20:2(n-6)	-26.96 \pm 0.61 ^a	-25.84 \pm 2.23 ^a
Docosadienoic acid	C22:2(n-6)	-27.12 \pm 0.17^a	-22.99 \pm 0.19^b
Linoleic acid	C18:2(n-6)	-28.19 \pm 0.85 ^a	-29.56 \pm 0.26 ^a
n-3 LCFAs			
Eicosatrienoate	C20:3(n-3)	-22.77*	-20.81
Docosaheptaenoic	C22:6(n-3)	-22.39 \pm 0.52^a	-23.98 \pm 0.36^b
Eicosapentenoic acid	C20:5(n-3)	-23.39 \pm 0.45 ^a	-23.60 \pm 0.67 ^a

*Only 1 replicate

**Only 2 replicates

Table 4.5 Mean $\delta^{13}\text{C}$ values ($\text{‰} \pm \text{SEM}$) for fraction of phospholipids extracted from degutted postlarval whiteleg shrimp ($n = 3$ or 4). Different letter superscript indicates significant differences ($P \leq 0.05$).

Long-chain fatty acid groups (LCFAs)	Carbon:Double bond (C:D)	0 hr	24 h
Saturated LCFAs			
Myristic acid	C14:0		-1.40; 5.68**
Pentadecanoic acid	C15:0		39.37; 54.83**
Palmitic acid	C16:0	-22.19 \pm 0.21^a	93.29; 110.46**
Heptadecanoic acid	C17:0		
Stearic acid	C18:0	-25.26 \pm 0.12^a	43.28 \pm 21.50^b
Arachidic acid	C20:0	-23.54 \pm 0.67	-16.44; -14.93**
Heneicosanoic acid	C21:0		-20.22*
Monounsaturated FAs			
Pentadecenoic acid	C15:1(n-5)	-15.97 \pm 2.17	2.19; 5.70**
Palmitoleic acid	C16:1(n-7)	-22.76 \pm 0.59	39.02; 37.78**
Heptadecenoic acid	C17:1(n-7)	-25.10 \pm 0.29^a	12.56 \pm 2.47^b
Vaccenic acid	C18:1(n-7)	-26.39 \pm 0.13^a	-19.60 \pm 0.66^b
Eicosenoic acid	C20:1(n-9)	-26.93 \pm 0.70 ^a	-23.77 \pm 2.27 ^a
n-6 LCFAs			
Arachidonic acid	C20:4(n-6)	-26.37 \pm 0.44^a	-16.58 \pm 1.80^b
Dihomo-gamma-linolenic acid	C20:3(n-6)	-24.04 \pm 0.19^a	-23.66 \pm 0.41 ^a
Ecosadienoic acid	C20:2(n-6)	-23.29 \pm 0.31	
Docosadienoic acid	C22:2(n-6)	-23.22 \pm 0.18 ^a	-23.76 \pm 0.26 ^a
n-3 LCFAs			
Eicosapentenoic acid	C20:5(n-3)	-26.20 \pm 0.23 ^a	-22.03 \pm 2.26 ^a
Eicosatrienoate	C20:3(n-3)	-23.61 \pm 0.77^a	-17.97 \pm 1.39^b

*Only 1 replicate

**Only 2 replicates

Table 4.6 ^{13}C enriched-FAs* and their corresponding contents (expressed in area %) in various lipid fractions extracted from degutted postlarval whiteleg shrimp. Values are means (either having 3 or 4 replicates) \pm SEM.

Long-chain fatty acid groups (LCFAs)	Carbon:Double bond (C:D)	Neutral lipids		Glycolipids		Phospholipids	
		Area %	δ ¹³ C	Area %	δ ¹³ C	Area %	δ ¹³ C
Saturated LCFAs							
Palmitic acid	16:00	14.76; 17.49**	16.32 ± 1.73	25.02 ± 0.56	21.78 ± 1.15		
Stearic acid	18:00			17.14 ± 0.80	-15.95 ± 3.04	9.27±0.32	43.28 ± 21.50
Monounsaturated LCFAs							
Palmitoleic acid	16:1(n-7)			1.70 ± 0.07	83.32 ± 8.58		
Heptadecenoic acid	17:1(n-7)					0.25±0.00	12.56 ± 2.47
Oleic acid	18:1(n-9)	15.71; 20.86**	-22.01 ± 0.68	24.65 ± 0.43	-20.91 ± 0.80		
Vaccenic acid	18:1(n-7)	3.33; 4.04**	53.34 ± 7.90	4.26 ± 0.13	38.13 ± 4.38	3.01±0.06	-19.60 ± 0.66
Erucic acid	22:1(n-9)			0.21 ± 0.05	-22.99 ± 0.19		
n-6 LCFAs							
Arachidonic acid	20:4(n-6)	0.14; 0.47**	-21.71 ± 1.77			3.84±0.10	-16.58 ± 1.80
Docosadienoic acid	22:2(n-6)				-22.99 ± 0.19		
n-3 LCFAs							
Eicosatrienoate	20:3(n-3)					0.17±0.00	-17.97 ± 1.39

*Only FAs that were significantly enriched with ^{13}C at 24 h relative to 0 h were considered.

**Only 2 replicates (separated by semi-colon).

Table 4.7 compares the ^{13}C enrichment between diet supplemented with ^{13}C -labeled *R. eutropha* DSM545 and LCFAs in various lipid fractions of postlarval whiteleg shrimp. The saturated LCFAs pentadecanoic acid (15:0) and palmitic acid (16:0) were enriched in the diet and in all the lipid fractions of the postlarvae. The LCFAs myristic acid (14:0) and stearic acid (18:0) which were enriched in the diet were also enriched in both glyco- and phospholipid fractions but either non-enriched or not observed in the neutral lipids. The LCFA heptadecanoic acid (17:0) was also enriched in the diet and in the neutral and glycolipid fractions of the postlarvae but not in the phospholipid fraction. The LCFA docosanoic acid (22:0), on the otherhand, was enriched in the diet but not in any of the lipid fractions of the postlarvae. For the monounsaturated LCFAs, pentadecenoic acid (15:1 (n-5)), palmitoleic acid (16:1 (n-7)), heptadecenoic acid (17:1 (n-7)) and vaccenic acid (18:1 (n-7)) were enriched in the diet. These were also enriched in all the lipid fractions of the postlarval whiteleg shrimp, however, no statistical support can be made due to insufficient number of replicates. The LCFA oleic acid 18:1 (n-9) did not show enrichment in the phospholipid while this was enriched in the diet and in both neutral and glycolipid fractions of the postlarvae. The LCFAs myristoleic acid (14:1 (n-5)) and nervonic acid (24:1 (n-9)) were enriched in the diet but not in any lipid fractions of the postlarvae. The LCFA erucic acid 22:1 (n-9) which was not enriched in the diet showed enrichment in the glycolipid but not in the other two lipid fractions of the postlarval whiteleg shrimp. For the n-6 LCFAs, arachidonic acid (20:4 (n-6)) and dihomo-gamma-linolenic acid 20:3 (n-6) showed enrichment in the diet and in one or two lipid fractions of the postlarvae whereas linoleic acid (18:2 (n-6)) which was also enriched in the diet was not enriched in any of the

lipid fractions of the postlarvae. For the n-3 LCFAs, eicosapentaenoic acid (20:5 (n-3)) showed enrichment in the diet but not in any of the lipid fractions while eicosatrienoate (20:3 (n-3)) in the glyco –and phospholipid fractions were enriched but not in the neutral lipid fraction or in the diet. Overall, there was a trend of lower ^{13}C enrichment in the lipid fractions of the postlarval whiteleg shrimp relative to the ^{13}C enrichment in the diet.

Table 4.7 Comparison between ¹³C enrichment in diet supplemented with ¹³C-labeled *R. eutropha* DSM545 and in various lipid fractions of degutted postlarval whiteleg shrimp.

Long-chain fatty acid groups (LCFAs)	Carbon:Double bond (C:D)	Diet	Lipid fractions in postlarval whiteleg shrimp		
			Neutral lipids	Glycolipids	Phospholipids
Saturated LCFAs					
Myristic acid	14:00	+++	x	‘+’	+
Pentadecanoic acid	15:00	++++	+	++	++
Palmitic acid	16:00	+++	++	++	+++
Heptadecanoic acid	17:00	+++	‘+++’	‘+++’	x
Stearic acid	18:00	‘+’	-	‘++’	++
Docosanoic acid	22:00	‘++’	x	x	x
Monounsaturated LCFAs					
Myristoleic acid	14:1(n-5)	++++	x	x	x
Pentadecenoic acid	15:1(n-5)	+++++	‘+++’	++	+
Palmitoleic acid	16:1(n-7)	+++	++	++	++
Heptadecenoic acid	17:1(n-7)	+++++	++	+++	++
Oleic acid	18:1(n-9)	‘++’	‘+++’	‘+++’	x
Vaccenic acid	18:1(n-7)	+++	++	++	‘++’
Erucic acid	22:1(n-9)	x	x	‘++’	x
Nervonic acid	24:1(n-9)	+++	x	x	x
n-6 LCFAs					
Linoleic acid	18:2(n-6)	‘++’	-	-	x
Arachidonic acid	20:4(n-6)	++++	‘++’	-	‘++’
Dihomo-gamma-linolenic acid	20:3(n-6)	++	x	‘+++’	-
Docosadienoic acid	22:2(n-6)	x	x	‘++’	-

n-3 LCFAs

Eicosatrienoate	20:3(n-3)	x	x	‘+++’	‘+ +’
Eicosapentaenoic acid	20:5(n-3)	+ +	-	-	-
Docosahexaenoic acid	22:6(n-3)		-	-	

Legends:

+	$\delta^{13}\text{C}$ value presented as ones
+	$\delta^{13}\text{C}$ value presented as ones (no statistical support because of insufficient number of replicates)
+ +	$\delta^{13}\text{C}$ value presented as tens
+	$\delta^{13}\text{C}$ value presented as tens (no statistical support because of insufficient number of replicates)
+ + +	$\delta^{13}\text{C}$ value presented as hundreds
+	$\delta^{13}\text{C}$ value presented as hundreds (no statistical support because of insufficient number of replicates)
+ + + +	$\delta^{13}\text{C}$ value presented as thousands
+ + + + +	$\delta^{13}\text{C}$ value presented as tens of thousands
‘+ / + +’	$\delta^{13}\text{C}$ value in negative
‘+ / + +’	$\delta^{13}\text{C}$ value in negative (no statistical support because of insufficient number of replicates)
-	Non-enriched FAs
x	No $\delta^{13}\text{C}$ value obtained

4.4 Discussion

In this study, the measurement of the stable carbon isotope ratio ($\delta^{13}\text{C}$) was applied to determine the assimilation of PHB offered in its natural matrix to two crustacean species, postlarval whiteleg shrimp *L. vannamei* and *Artemia* sp.

According to Situmorang (2015), the production of PHB in the bacterial strain *R. eutropha* DSM545 is triggered by nutrient limitation (particularly ammonium) combined with the provision of ^{13}C -labeled glucose to the cultivation broth. This procedure is intended to redirect the carbon flux from production of bacterial biomass to PHB synthesis, typically from 10 % to 75 % (Mozumder *et al.* 2014). Based on this information, it is expected that there was a massive transfer of the ^{13}C signal originating from the labeled glucose to the PHB. The data also show that the ^{13}C may be partly transferred to the structural components (e.g. FAs) of the bacterial cell. This is based on the consistent enrichment of the two LCFAs pentadecanoic acid (15:0) and palmitic acid (16:0) in the ^{13}C -enriched shrimp diet and in the postlarval whiteleg shrimp. These LCFAs are reported to be two of the most dominant saturated FAs in *Ralstonia* spp. For instance, Behiry *et al.* (2015) reported the LCFAs pentadecanoic acid (15:0) and palmitic acid (16:0) to account for 13 and 48 % and 18 and 22 %, respectively, of the total FA contents in two European *R. solanacearum* isolates Scotland RsSc1 and NetherlandsRsNe1, respectively. In the present study, however, the exact ratio of the ^{13}C transferred in PHB and in the structural components of *R. eutropha* DSM545 is not known. This would warrant further investigation.

The LCFA composition of diet supplemented either non-labeled or ^{13}C -labeled *R. eutropha* DSM545 closely reflect each other indicating that the provision of ^{13}C -labeled glucose during the PHB-inducing period as abovementioned did not influence the contents of LCFAs, although these values were not statistically tested. The ^{13}C enrichment in LCFAs from various lipid fractions of the postlarval whiteleg shrimp was in general term lower than in the shrimp diet, indicating that the PHB present in natural matrix (e.g. contained in a bacterial carrier) may not be used as a building block but probably rather as energy source as previously mentioned by De Schryver *et al.* (2010a) and Defoirdt *et al.* (2007b).

There was also an indication that ^{13}C enrichment of LCFAs was not necessarily linked to its abundance. For example, the two most highly enriched LCFAs pentadecenoic (15:1(n-5)) and heptadecenoic acid (17:1(n-7)) in the diet showed a mean $\delta^{13}\text{C}$ value of approximately 96 000 ‰ and 29 000 ‰, respectively, but these LCFAs constituted only 0.03 and 0.38 in area %, respectively, of the total FA contents in the diet. On the otherhand, the three most abundant LCFAs palmitic acid (16:0, approx. 16 area %), oleic acid (18:1(n-9), approx. 19 area %) and linoleic acid (18:2(n-6), approx. 35 area %) showed a mean $\delta^{13}\text{C}$ values of only 300, -21 and -21 ‰, respectively. Basically, these data show that major FAs found in the diet can be lowly enriched while minor FAs can be highly enriched with ^{13}C . This pattern was also observed for the postlarval whiteleg shrimp, although LCFAs may differ from the ones mentioned in the diet.

Interestingly, the LCFAs erucic acid (22:1 (n-9)), docosadienoic acid (22:2 (n-6)) and eicosatrienoate (20:3(n-3)) were enriched in one of the lipid fractions of the postlarval whiteleg shrimp but not in the diet. This indicates the capacity of the postlarvae to elongate certain FAs. The capacity of crustaceans to bioconvert FAs was also demonstrated by De Troch *et al.* (2012) using harpacticoid copepods. These authors have measured arachidonic acid (20:4 (n-6)) in the copepod while this was missing from the bacterial diet. In the present study, the presence of erucic acid (22:1 (n-9)) in the glycolipid fraction of the postlarvae may have resulted as an elongation product of oleic acid (18:1 (n-9)) which was present in the diet (Akoh & Min 1998) while docosadienoic acid (22:2 (n-6)) may have resulted from the subsequent elongation of linoleic acid (18:2(n-6)) in the diet as this FA is known to be the metabolic precursor of all the FAs of the n-6 series (Patterson *et al.* 2012). On the otherhand, eicosatrienoate (20:3(n-3)) is usually an elongation product of alpha-linolenic acid (18:3(n-3)) (British Nutrition Foundation 1992) however, the eicosatrienoate (20:3(n-3)) in the phospholipid fraction of the postlarvae may have come from elongation or desaturation systems other than alpha-linolenic acid (18:3(n-3)) as this FA was not detected in the diet. In the case of *Artemia*, there was no indication whether the high ^{13}C assimilated in its tissue ended up in its FA components.

The potential interference of diet composition on ^{13}C enrichment was also explored in the present study. The ^{13}C enrichment in postlarval whiteleg shrimp fed low or high fat diet did not significantly differ. Findings in *Artemia* nauplii fed ^{13}C -labeled *R. eutropha* DSM545 alone or in combination with *T. suecica*, *D. tertiolecta* or LVS3 also resulted in an overall non-significantly different $\delta^{13}\text{C}$ values. These results indicate that assimilation

of PHB in natural matrix is not influenced by dietary composition, possibly allowing PHB to fully exert its beneficial effects on the aquatic farmed host. In the case of *Artemia*, the significantly low $\delta^{13}\text{C}$ in treatment Art + *T. suecica* + ^{13}C -labeled *R. eutropha* DSM545 as compared to Art + LVS3 + ^{13}C -labeled *R. eutropha* DSM545 may indicate that *T. suecica* is the preferred feed item over LVS3. Findings of Makridis & Vadstein (1999) have shown higher preference of *Artemia* for particles with a diameter of 4-8 μm compared to smaller particles ($\leq 1 \mu\text{m}$). The microalga *T. suecica* and the bacterial strain LVS3 have an average cell diameter of 8.5 μm (Lananan *et al.* 2013) and $< 1 \mu\text{m}$ (Jendrossek *et al.* 2007; Makridis & Vadstein 1999), respectively. *T. suecica* may also be a better feed for *Artemia* than LVS3 due to its nutritional properties such as significant source of LCFAs (Brown 2002) and lutein (Rønnestad *et al.* 1998).

It is also noteworthy to mention that ^{13}C seemed to be rapidly assimilated in the tissues of postlarval whiteleg shrimp based on the trend of higher $\delta^{13}\text{C}$ value at 4 h relative to 0 h irrespective of the diet. Similarly, significantly higher $\delta^{13}\text{C}$ value was seen in *Artemia* nauplii after 2 h as compared to 0 h. These findings may indicate that PHB offered in natural matrix is rapidly assimilated in the tissues of crustaceans. Earlier results obtained by Situmorang (2015) on Nile tilapia *O. niloticus* fed diet supplemented with ^{13}C -labeled *R. eutropha* DSM545 containing 75 % of PHB on DW, showed a non-significantly different $\delta^{13}\text{C}$ values at 2 h until 24 h relative to 0 h. In the faecal matter, however, these authors have found significantly higher $\delta^{13}\text{C}$ value at 2 h relative to 0 h indicating that majority of the ^{13}C at this time point was not transferred to the tissues of the Nile tilapia

but excreted as waste product. The difference in the results obtained in the present study and the earlier study may be species-dependent.

In conclusion, this study is the first to demonstrate the assimilation of PHB in crustacean tissues. There was a trend of lower ^{13}C enrichment in LCFAs from various lipid fractions of the postlarval whiteleg shrimp than in the bacterial lipids indicating that PHB offered in natural matrix could not be used a building block but probably rather as energy source. Furthermore, assimilation of PHB offered in natural matrix was not influenced by dietary feed composition and it is rapidly assimilated in the tissues of the crustacean species. For future work, it is recommended to investigate the assimilation of PHB offered in natural matrix in short chain FAs (SCFAs) of crustacean species as literature has mentioned that PHB is degraded into short chain FAs (SCFAs; C2-C6) (Defoirdt *et al.* 2009).

Acknowledgment

The authors would like to thank Dirk van Gansbeke of the Department of Biology of Ghent University Campus Sterre-S8 and Geert Vandewiele of the Laboratory of Aquaculture and *Artemia* Reference Center (ARC) (Ghent University, Belgium) for the analysis of the samples.

Chapter 5

Application of poly- β -hydroxybutyrate (PHB)-based bioplastic as artificial substratum in penaeid shrimp culture

Chapter 5

APPLICATION OF POLY- β -HYDROXYBUTYRATE (PHB)-BASED BIOPLASTIC AS ARTIFICIAL SUBSTRATUM IN PENAEID SHRIMP CULTURE

Gladys Ludevese-Pascual¹, Joseph Laranja^{1, 2}, Edgar Amar², Peter Bossier^{1*} & Peter De Schryver^{1*}

¹ Laboratory of Aquaculture & *Artemia* Reference Centre, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University Rozier 44, B-9000 Gent, Belgium

²Southeast Asian Fisheries Development Centre/Aquaculture Department (SEAFDEC/AQD), Brgy. Buyuan, Tigbauan Iloilo 5021 Philippines

*Shared senior authorship

Abstract

The application of artificial substratum in penaeid shrimp culture units has been shown beneficial in terms of increasing culture performance of the farmed animals and improving water quality. In the present study, the use of artificial substratum made up out of poly- β -hydroxybutyrate (PHB) based biodegradable plastic for penaeid shrimp culture was investigated. The survival of postlarval tiger shrimp *Penaeus monodon* (30 ± 5 mg) provided with PHB substratum made out of PHB type DP9002 (Metabolix GmbH, Köln, Germany) was 88.7 ± 3.4 % and this was significantly higher as compared to postlarvae provided conventional substratum consisting of polyvinylchloride (PVC) pipes (67.3 ± 6.5 %). In a separate experiment, provision of PHB substratum made out of PHB type P209

(Biomer, Krailling, Germany) to postlarval whiteleg shrimp *Litopenaeus vannamei* (106 ± 52 mg) also resulted in significantly higher survival of 83.6 ± 3.4 % as compared to 73.9 ± 3.0 % for postlarvae provided with PVC substratum. Results on final weight showed a significantly higher weight of $1\,008.2 \pm 4.5$ mg in postlarval whiteleg shrimp provided PHB substratum as compared to 893.4 ± 30.0 mg for postlarvae provided with PVC substratum. However, no significant weight improvement was observed for the postlarval tiger shrimp indicating species-dependent effect of PHB on growth. Nevertheless, a trend of improved robustness against adverse environmental conditions (lethal ammonium chloride concentration) and increased resistance to pathogenic *Vibrio* was observed in postlarval tiger shrimp provided with PHB substratum as compared to postlarvae provided with PVC substratum. An additional test was conducted to evaluate the effect of artificial substratum on water quality. A trend towards higher total ammonia-nitrogen (TAN) conversion from an initial concentration of 1.75 ± 0.0 mg L⁻¹ to 0.35 ± 0.04 mg L⁻¹ after 72 h was observed using PHB substratum while using no substratum and PVC substratum lead to TAN concentrations of 1.28 ± 0.06 mg L⁻¹ and 1.23 ± 0.10 mg L⁻¹, respectively. The level of total inorganic nitrogen (TIN) after 72 h was significantly lower for PHB substratum (1.25 ± 0.27 mg L⁻¹) as compared to either no substratum (2.17 ± 0.18 mg L⁻¹) or PVC substratum (2.27 ± 0.19 mg L⁻¹). Overall, this study indicates that artificial substratum consisting of PHB-based biodegradable plastic increases the quality of postlarval penaeid shrimp and that it contributes to maintaining good water quality.

5.1 Introduction

The steady increase in shrimp production by aquaculture is the consequence of improved methods and techniques related to hatching and rearing of shrimp larvae and postlarvae (Thompson *et al.* 1999). Among the methods and techniques introduced in shrimp farming is the provision of artificial substratum. Substratum generally refers to non-living materials on which an animal lives or fixed organism is attached. The use of artificial substratum in shrimp culture systems is thought to overcome the negative effects of increased stocking density on growth and survival such as the decrease in favorable space and natural food sources (Kautsky *et al.* 2000; Arnold *et al.* 2006; Zhang *et al.* 2010). Indeed, Zhang (2011) found significantly higher mean weight (13 g) and survival (88 %) in whiteleg shrimp *Litopenaeus vannamei* postlarvae offered artificial substratum composed of polypropylene fabric screens that was immersed in the culture water all the time as compared to postlarvae offered no artificial substratum (mean weight and survival of 6.6 g and 59 %, respectively). In postlarval *Penaeus monodon* stocked at a density of 5 000 shrimp m⁻³, a trend of higher survival, individual weight and harvest density of 61 %, 0.40 g and 2 956 shrimp m⁻³, respectively, were seen in postlarvae supplemented with artificial substratum composed of non-buoyant AquaMat as compared to postlarvae offered no artificial substratum with survival, individual weight and harvest density of 49 %, 0.20 g and 2 410 shrimp m⁻³, respectively (Arnold *et al.* 2009). In another study, improved weight (from an average of 9.5 g to an average of 13.4 g final weight) and overall improved production (from 1 342 kg ha⁻¹ to 2 404 kg ha⁻¹) were observed for freshwater

prawn *Macrobrachium rosenbergii* juveniles in case substratum was added in the culture system (Tidwell & Coyle 2008).

Several studies have discussed the importance of artificial substratum in controlling water quality (Zhang *et al.* 2016; Pradeep *et al.* 2003, Azim *et al.* 2002; Tidwell *et al.* 1998). Nitrogenous compounds such as ammonia and nitrite resulting from excess feed and excretory product often cause deterioration of water quality in hatchery system (Chen & Lei 1990). Thus, most hatchery operators resort to daily water exchange to maintain acceptable water quality for larval rearing (Khatoon *et al.* 2007a). However, frequent water exchange to overcome poor water quality problems in tanks is laborious and expensive (Thompson *et al.* 2002) and discharge of large amount of waste water from hatcheries may also cause eutrophication in rivers and coastal waters (Ziemann *et al.* 1992). Because of these, an alternative strategy implying the use of a substratum-based culture has been introduced. The application of artificial substratum either coated with periphytic cyanobacterium (*Oscillatoria* sp.) or mixed diatoms (*Amphora* sp., *Navicula* sp. and *Cymbella* sp.) was shown to improve the water quality in the culture of *P. monodon* postlarvae. Tanks with *Oscillatoria*-coated substratum had a total ammonia-nitrogen (TAN), nitrite–nitrogen (NO_2^- -N) and soluble reactive phosphorus (SRP) concentrations of 0.03 mg L^{-1} , 0.01 mg L^{-1} and 0.05 mg L^{-1} , respectively, while these were 0.82, 0.52 and 0.35 mg L^{-1} , respectively, in tanks containing mixed diatoms-coated substratum. These values were significantly lower as compared to a treatment without substratum (1.14, 0.80 0.53 mg L^{-1} of TAN, NO_2^- -N and SRP, respectively) (Khatoon *et al.* 2007b). In juvenile *L. vannamei*, provision of artificial substratum (Aquamats) previously submerged in an

operating shrimp pond for 5 days to allow formation of biofilm, was found to significantly lower the ammonia-nitrogen ($\text{NH}_3\text{-N}$) from 0.18 in a treatment offered no artificial substratum to 0.04 mg L^{-1} in treatment with artificial substratum (Audelo-Naranjo *et al.* 2012). The improvement of water quality in substratum-based culture is said to be attributable to the microbial community associated with the attached biofilm. According to Ootoshi *et al.* (2006), artificial substratum can create habitat for nitrifying and denitrifying bacteria known to convert toxic nitrogenous wastes into less toxic forms. Commonly used artificial substrata in shrimp culture systems include fibreglass window screens (Sandifer *et al.* 1987), plastic mesh and oyster netting (Tidwell *et al.* 1999) and polyvinylchloride (PVC) frames and pipe (Tidwell *et al.* 1998; Khatoon *et al.* 2007b). Other types of artificial substrata such as bamboo pipe, plastic sheet, fibrous scrubber and ceramic tiles were introduced by Khatoon *et al.* (2007a) in nutrient enriched brackishwater shrimp ponds. Despite the noted positive contribution of using artificial substratum in shrimp culture, several disadvantages are known. Among these are the tendency of biofouling with polychaetes (Khatoon *et al.* 2007a) for example, the eunicid polychaete, *Marphysa graveleyi*, that could serve as passive vectors of white spot syndrome virus (WSSV) in the transmission of white spot disease to *P. monodon* broodstocks (Vijayan *et al.* 2005), and interference during harvesting (eg twigs, pipes, bricks) (FAO 2002b). Perhaps the most significant problem is the use of artificial substratum made up of non-biodegradable plastic (i.e. PVC) that are problematic to dispose after its usage (Lithner 2011). Hence, suitable substratum that would favor both the farmed aquatic animals and the environment would be beneficial.

Increasing environmental pollution and exhaustion of non-renewable fossil resources have encouraged research on biosynthetic and biodegradable materials (Matavulj & Molitoris 1992). Novel biodegradable plastics include polyhydroxyalkanoates (PHAs) (Garcia *et al.* 1999), which are naturally occurring polyesters synthesized by bacteria of different genera. The molecular mass of most bacterial PHAs varies but generally is in the order of 50 000 to 1 000 000 daltons which are sufficiently high to have a polymeric character (Madison & Huisman 1999). The application of PHAs has gained considerable attention due to their biodegradability, making them extremely desirable substitutes for synthetic plastics (Kalia *et al.* 2007). The homopolymer, poly- β -hydroxybutyrate (PHB), the most common PHA, is completely degraded into carbon dioxide and water through natural microbiological mineralization (Braunegg *et al.* 1998). PHB-based bioplastics have been widely used for the manufacturing of medical devices such as scaffolds and artificial esophagus and in tissue engineering (Chang *et al.* 2014). In penaeid aquaculture systems, the application of PHB either as enrichment product or as food additive has also been tested and reported to result in promotion of growth (Nhan *et al.* 2010; Sui *et al.* 2012), improved robustness against adverse environmental conditions (Laranja *et al.* 2014) and enhanced resistance against pathogens (Defoirdt *et al.* 2007b; Sui *et al.* 2012; Halet *et al.* 2007; Laranja *et al.* 2014; Thai *et al.* 2014). It is also regarded as energy source (De Schryver *et al.* 2010a; Defoirdt *et al.* 2007b). However, there have been no reports described on the application of PHB-based bioplastic as artificial substratum. The goal of the present study is therefore to evaluate the application of PHB-based bioplastic as artificial substratum during culture of penaeid shrimp. Its beneficial effects on the culture

performance of the postlarvae of two shrimp species - tiger shrimp and whiteleg shrimp - and its contribution in improving their robustness against adverse environmental conditions and pathogenic infection are evaluated to determine whether PHB offered as substratum could also result in a similar way when offered as feed additive and/or enrichment product. Literature reports have also mentioned of the contributions of artificial substratum in improving water quality in shrimp culture systems (Khatoon *et al.* 2007b; Audelo-Naranjo *et al.* 2012) and thus, the capacity of PHB substratum to maintain good water quality as well as its overall attractiveness towards postlarval penaeid shrimp are also considered.

5.2 Materials and Methods

5.2.1 Tiger shrimp P. monodon postlarvae

A first experiment was performed using tiger shrimp *P. monodon* postlarvae. Postlarvae (PL12) were obtained from the Shrimp facility of the Southeast Asian Fisheries Development Centre/Aquaculture (SEAFDEC/AQD) (Tigbauan, Iloilo Philippines) and transported to the laboratory according to the procedures described in Chapter 2 (section 2.2). Upon arrival, postlarvae (30 ± 5 mg) were stocked at 1 individual L⁻¹ in three cylindroconical acclimation tanks of 250 L filled with 200 L UV-treated seawater. Ten PVC pipes (10 cm long, 2.54 cm diameter, 0.25 cm thickness) were provided in each acclimation tank as shelter. The seawater used was passed through a series of filter cartridges (5, 10 and 15 µm) before being passed through an ultraviolet filter at a capacity of approximately 30 L min⁻¹ (Mega Fresh, Taiwan). During the 14-day acclimation period,

postlarvae were fed SEAFDEC-formulated diets (Table 5.1) containing 45 % crude protein and 9 % crude fat at a level of 5 % on wet body weight (BW). The daily ration was divided into equal amounts and offered two times a day (9:00 and 16:00 h). Temperature and salinity in the acclimation tanks were 30 ± 2 °C and 32 g L⁻¹, respectively. Aeration by means of an air diffuser was provided to assure levels of dissolved oxygen at ≥ 5 mg L⁻¹ and water exchange was administered every two days at 25 % of the water volume to remove waste compounds. A linear fluorescent lamp (40 W, Philips, USA) was used to give a daily light regime of 10 h light and 14 h dark.

Table 5.1 Dietary feed composition.

Ingredients	% composition on dry weight (DW)
Danish fishmeal	33.0
Squid liver powder	10.0
Dehulled soybean meal (DSBM)	7.0
<i>Acetes</i> sp.	15.0
Wheat Pollard	2.5
Bread flour	13.0
Soybean oil	1.0
Squid oil	2.0
Peruvian fish oil	2.0
Lecithin	0.5
Vitamin mix	2.0
Mineral mix	2.0
Tasmix	0.5
Choline chloride	0.2
Vitamin C	0.1
Dicalphos	4.0
Cellulose	5.3

5.2.2 Whiteleg shrimp *L. vannamei* postlarvae

Experiment 2 was performed with whiteleg shrimp postlarvae. Postlarvae (PL10; 106 ± 52 mg) were obtained from Shrimp Improvement Systems (Islamorada, Florida) and maintained in a recirculating system of the Shrimp Facility of the Laboratory of Aquaculture and *Artemia* Reference Center (ARC) (Ghent University, Belgium) until PL30. The rearing conditions in the recirculating system were described in Chapter 4 (section 4.2).

5.2.3 Artificial substrata

PHB-based bioplastic sheets made out of PHB type DP9002 (0.05 cm wall thickness) were obtained from Metabolix GmbH (Köln, Germany) and PHB-based bioplastic sheets made out of PHB type P209 (0.03 cm wall thickness) were obtained from Biomer (Krailling, Germany) for use in Experiment 1 and 2, respectively. Both PHB bioplastics were company blends consisting of PHB supplemented with undefined plasticizers to improve its flexibility. The PHB sheets were rolled into a cylindrical shape (10 cm long, 2.54 cm diameter) and fixed by the use of a plastic cable tie. Commercially-available PVC pipes of the same dimensions were used as the control substratum. Both PHB and PVC were abraded using sandpaper until achieving the same roughness.

One artificial substratum unit consisted of three rolls of substratum joined together in a pyramidal-shape. All artificial substrata units were first allowed to age for 15 days in a shrimp tank containing adult *P. monodon* of the Shrimp Facility of SEAFDEC/AQD (Experiment 1) or in a recirculating tank of the Shrimp Facility of ARC containing no

shrimps but provided directly with water from the biofilter (Experiment 2). PHB substratum in Experiment 1 was wrapped with a polyester netting (2 mm mesh size) during the aging to prevent direct contact with *P. monodon*. The “aging” resulted in biofilm formation on the surface of the artificial substrata. As the aged PHB substratum used in Experiment 1 showed cracks, these were held together in an aluminum wire basket (12 cm length x 7 cm width x 1 cm height). This was also done for the control substratum consisting of PVC pipes.

Finally, the ‘aged’ substrata were transferred to a fiberglass tank for 3 days prior to the actual experiment. These tanks were provided with black cover to prevent light penetration and a small amount of ammonium chloride (1 mg L^{-1} ; Sigma Aldrich) was supplemented daily to sustain the growth of the biofilm.

5.2.4 Experiment 1: Use of PHB substratum for tiger shrimp postlarvae culture

5.2.4.1 Experimental design

Experiment 1 consisted of 60 L fiberglass tanks filled with 60 L UV-treated seawater that was passed through a series of filter cartridges as mentioned in section 5.2.1. Temperature and salinity of the water were $30 \pm 2 \text{ }^{\circ}\text{C}$ and 32 g L^{-1} , respectively. The tanks were stocked with the acclimated shrimp postlarvae at a density of 1 individual L^{-1} and contained one of the following substrata:

- PVC substratum ($n = 5$ tanks)
- PHB-based substratum (Metabolix GmbH) ($n = 5$ tanks)

The substratum in each tank consisted of 2 aluminum baskets carrying the ‘aged’ material. Postlarvae were fed at 9:00 and 16:00 h with diets described in section 5.2.1 at 5 % on wet BW per day. Each experimental tank was supplied with air through an air diffuser to maintain dissolved oxygen above 5 mg L⁻¹ and the light regime was set at a fixed 10 h light and 14 h dark. The total ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen concentrations were monitored daily in 5 randomly selected tanks using JBL test kits (Neuhofen, Germany). Once the level reached a critical value of 0.2 mg L⁻¹ total ammonia-N, 0.1 mg L⁻¹ nitrite-N or 10.0 mg L⁻¹ nitrate-N in any one of the measured tanks, 50 % of the water was replaced in all experimental tanks. The pH, temperature, salinity and dissolved oxygen in all experimental tanks were monitored daily using YSI 556 MPS Multiprobe System (Japan). The experimental trial lasted for 61 days.

5.2.4.2 Measured parameters

The overall status of the postlarvae was examined according to the methods described in Chapter 2 (2.2.1). Survival and growth of the postlarvae were evaluated at the end of the experimental trial. Survival was determined by counting the number of animals in each tank. The percentage survival was computed following the formula of Thompson & Bergersen (1991):

$$\text{Survival (\%)} = \left(\frac{X}{N} \right) * 100$$

where X is the number of larvae present at the end of the experimental period and N is the number of larvae at stocking (day 0). The mean weight within each replicate tank was determined by collecting all surviving postlarvae that were not used in the stress test and

challenge trial. These were sacrificed by immersion in ice cold seawater, blotted dry to remove adhering water and weighed individually on an analytical balance with 0.0001 g precision. Next, the mean weight for each treatment at the end of the experimental trial was computed as the mean of the average weights from the replicate tanks within each treatment. The mean daily growth (ADG) was calculated within each replicate tank as mentioned in weight measurement and the mean ADG for each treatment was calculated following the formula of Ravuru & Mude (2014):

$$ADG = \frac{\text{Total wet body weight gained by the postlarvae}}{\text{Total days of culture}}$$

5.2.4.3 Stress test

The resistance against lethal ammonium chloride of the postlarvae at the end of the feeding trial was evaluated. The preliminary test – as explained in Chapter 2 (2.2.5) - indicated an LD₅₀ of ammonium chloride at 143 mg L⁻¹. The stress test was carried out according to Reed & Muench (1938) with slight modifications. Ten postlarvae were randomly collected from each tank and transferred to a container containing 2 L UV-sterilized seawater with 143 mg L⁻¹ ammonium chloride with a mean pH of 8.2. Air was provided into each container using an airstone diffuser. Test animals were not fed 12 h prior to the test and during the exposure period. The number of dead postlarvae was recorded at different time points (0, 24, 48, 72 and 99 h). Death was assumed when larvae were immobile and showed no response to external stimulus such as picking or touching with forceps.

5.2.4.4 *Vibrio* challenge test

The resistance against pathogenic infection of the postlarvae at the end of the feeding trial was evaluated. Ten postlarvae were randomly collected from each tank and immersed in a sublethal concentration of ammonium chloride (100 mg L^{-1} , pH of 8.2) for 24 h in containers containing 2 L UV-sterilized seawater. Each tank was individually aerated by means of an airstone diffuser. After exposure, postlarvae were transferred to a new tank containing 2 L of UV-sterilized seawater with $10^7 \text{ cell mL}^{-1}$ pathogenic *Vibrio campbellii* LMG 21363. Preparation of the bacterial strain was done according to Defoirdt *et al.* (2007b). Postlarvae were given supplementary feeds once daily at 2 % on wet BW. No water exchange was administered all throughout the challenge test. Survival (%) was recorded every 24 h for 10 days.

5.2.4.5 *Substratum* visit test

An experiment to evaluate the attractiveness of the different substratum types was performed using postlarvae of the same original batch as the experimental postlarvae but that were not exposed to the substratum. After the main substratum trial on tiger shrimp, ten postlarvae ($500 \pm 10 \text{ mg}$) were introduced in the same experimental tank containing 60 L UV-treated seawater and one set of aluminum basket containing 3 rolls of either PVC or PHB substratum (now 76-day old starting from aging until end of main substratum trial) was provided ($n = 5$ tanks per substratum type). These postlarvae were deprived of food during 24 h prior to the actual test. The substratum was positioned on one side of the experimental tank while the postlarvae were released on the other side. Visit of substratum by postlarvae was determined as the proportion of postlarvae in the tank that were located

on the substratum and this was monitored at different time points (0, 1, 3, 6, 12, 24, 30, and 48 h) after introducing the postlarvae in the tank. Only postlarvae that were in direct contact with the substratum were considered and counted.

5.2.4.6 Lipid and fatty acid composition of the tiger prawn postlarvae

All surviving postlarvae from each tank replicate for each treatment were pooled to examine lipid and fatty acid contents at the end of the experimental trial. After measurement of weight, total lipid from tiger shrimp postlarvae was extracted and analyzed according to the method described by Folch *et al.* (1957) using freeze-dried samples (300 mg). Total lipid was expressed as percentage on dry weight (% on DW). Fatty acid analysis was performed according to methods described in Chapter 3 (3.2.5) and expressed either as mg fatty acid methyl esters (FAMES) per gram lipid (mg g^{-1} lipid) or mg FAME per gram dry weight (mg g^{-1} DW).

5.2.5 Experiment 2: Use of PHB substratum for whiteleg shrimp postlarvae culture

5.2.5.1 Experimental design

Experiment 2 consisted of 40 L fiberglass tanks filled with 40 L sand-filtered seawater. Temperature and salinity of the water were 28 °C and 35 g L⁻¹, respectively. The tanks were stocked with the postlarvae at a density of 1 individual L⁻¹ and contained one of the following substrata:

- PVC pipes ($n = 7$ tanks)
- PHB-based bioplastics ($n = 7$ tanks)

The substratum in each tank consisted of 3 sets (3 rolls set⁻¹) of the ‘aged’ material. Postlarvae were fed with commercial postlarval shrimp diet (CreveTec bvba, Ternat, Belgium) containing 54 % crude protein and 12 % crude fat at a level of 10 % on wet BW per day. The daily ration was divided into equal amounts and offered two times a day (9:00, 16:00 h). Feed ration was gradually reduced by 1 % week⁻¹ to a final ration of 5 % on wet BW per day. Each experimental tank was supplied with air through an air diffuser to maintain dissolved oxygen above 5 mg L⁻¹ and the light regime was set at a fixed 12 h light and 12 h dark. The levels of total ammonia-nitrogen, nitrite-nitrogen, and nitrate-nitrogen were measured on 7 randomly selected tanks on a daily basis as described in Experiment 1 section 5.2.4.1. Once the level of one of these parameters exceeded the acceptable level, 50 % of the rearing water in all tanks was changed with pre-heated seawater (28 °C). The experimental trial lasted for 55 days.

5.2.5.2 Measured parameters

The survival and growth of the postlarvae were evaluated at the end of the experimental trial following the methodology described in Experiment 1 section 5.2.4.2.

5.2.5.3 Substratum visit and preference

The attractiveness of the different substratum types by whiteleg shrimp postlarvae of the same original batch as the experimental postlarvae but that were not exposed to the substratum were conducted for non-aged (Test 1), 10-day aged (Test 2) and 70-day aged substrata (Test 3). The substrata in Test 1 were used directly after fabrication while substrata in Test 2 were allowed to age for 10 days in a recirculating shrimp tank

containing juvenile *L. vannamei* of the Shrimp Facility of ARC. Substrata for Test 3 were derived from the main substratum trials on whiteleg shrimp postlarvae. For each test, ten postlarvae were placed in 5 L transparent tanks filled with 4 L of pre-heated seawater (28 °C) and containing either PVC ($n = 5$ tanks) or PHB substratum ($n = 5$ tanks) for substratum visit test and both PVC and PHB substrata for substratum preference test ($n = 5$ tanks). The substratum consisted of 1 set (3 rolls) in test 1 and 2, while 1 set of both substratum types was used in test 3. All experimental tanks were placed in a water bath containing a heater (100 W, JBL Neuhofen Germany) to maintain water temperature in the tank at 28 °C. Air was provided into each experimental tank using an airstone diffuser and photoperiod was kept at 12 hours light and 12 hours dark. The postlarvae were deprived of food 24 h prior to the actual test. The visit and preference of the postlarvae were determined as the percentage of the postlarvae in the tank that were located on each of the substratum types and this was monitored at different time points (0, 3, 6, 9, 12, 15, 21 (or 18 in the case of test 1) and 24 h) after introducing the postlarvae in the tank. Only postlarvae that were in direct contact with the substratum were considered and counted.

5.2.6 Experiment 3: Effect of PHB substratum on water quality

The potential of the different substratum types in decreasing the total ammonia-nitrogen (TAN) in the water was evaluated. Tests were conducted in 5 L transparent tanks filled with 4 400 mL of pre-heated seawater (28 °C) and provided with either PVC substratum ($n = 3$ tanks) or PHB substratum ($n = 3$ tanks). A control treatment consisting of tanks without substratum ($n = 3$ tanks) was also included. The substratum consisted of

either 1 roll of PVC or an equivalent surface of PHB (to have identical surface to volume ratio as the PHB substratum already showed sign of degradation by the time of use in the experiment). In a first test, the nitrogen removal capacity of the different substratum types after single dosing to an initial level of 1.75 mg L^{-1} TAN with a mean water temperature and pH level of 28°C and 7.6 ppt, respectively, was investigated. Preparation of test solution was carried out according to the methods described by Najdegerami *et al.* (2015). In a second test, the nitrogen removal capacity of the different substratum types of nitrogen originating from whiteleg shrimp postlarvae ($1.03 \pm 0.03 \text{ g}$) at a density of 1 individual per L^{-1} stocked in each experimental tank was investigated. These postlarvae were fed the same experimental diet as in the main experiment on whiteleg shrimp. All experimental tanks were mounted in a water bath to maintain temperature at 28°C . Air was provided into each experimental tank using an airstone diffuser and photoperiod was kept at 10 h light and 14 h dark. During both tests, no water change was administered and the levels of TAN ($\text{NH}_3\text{-N} + \text{NH}_4^+\text{-N}$), nitrite-nitrogen ($\text{NO}_2^-\text{-N}$), nitrate-nitrogen ($\text{NO}_3^-\text{-N}$) and the sum of these parameters (total inorganic nitrogen; TIN) were measured at 0, 24, 48 and 72 h by means of a Multiparameter Bench Photometer HI 83203 (Hanna Instrument USA) using the modified Nessler, ferrous sulfate and cadmium reduction methods, respectively (American Society of the International Association for Testing and Materials 2000).

5.2.7 Statistical analysis

Descriptive statistics were used to assess normality of the data obtained on survival and growth at the end of the experimental trials. When the assumption of normality was achieved, data were analyzed using independent-sample T-tests. Data on survival at the

end of the experimental trial were arcsin transformed. The time-point survival data on stress and challenge tests were analyzed using logistic regression analysis (GenStat 16 VSN International, Hemel Hempstead, UK). The non-parametric Mann-Whitney U test was used to analyze data on substrata preference. All data presented in cumulative way were analyzed using repeated measures ANOVA (RM-ANOVA) and data sphericity was tested by means of the Mauchly's test. All statistical analyses were performed using SPSS Statistical software v. 11.5 unless otherwise stated and significance was set at an alpha level of $P \leq 0.05$. Graphic presentations of results were carried with Sigmaplot 13.0 (Systat Software, Inc., Chicago, IL, USA).

5.3 Results

5.3.1 Experiment 1: Use of PHB substratum for tiger shrimp postlarvae

5.3.1.1 Measured parameters

The experimental postlarvae were not infected with WSSV and IHHNV or at least the viral load was lower than the detection limit. Postlarvae were also found negative for MBV occlusion bodies, and muscular deformities were not detected indicating that the animals used in this experiment were physically fit at the start of the experiment.

The survival and growth of postlarval tiger shrimp at the end of the experimental trial are shown in Table 5.2. A significantly higher survival was seen for the postlarvae provided with the PHB substratum as compared to the PVC substratum ($t(8) = -2.948$, $P = 0.018$). There were no significant differences observed in mean body weight and mean

daily growth for postlarvae provided with the different substratum types at the end of the culture period.

Table 5.2 Survival (%) and growth of postlarval tiger shrimp provided either PVC or PHB substratum in the culture tank. Values represent means \pm SEM ($n = 5$). Different letters within a row indicate significant differences ($P \leq 0.05$; P -value in **bold**).

Parameters	Substratum types		<i>P</i> -value
	PVC	PHB	
Survival (%)	67.3 \pm 6.5 ^b	88.7 \pm 3.4 ^a	0.018
Mean wet body weight (mg)	572.9 \pm 66.4 ^a	504.8 \pm 23.8 ^a	0.602
Mean growth rate (mg day ⁻¹)	8.9 \pm 1.1 ^a	7.8 \pm 0.4 ^a	0.363

Figure 5.1 shows the weight frequency distribution of all the surviving postlarvae sampled in treatment provided either PVC or the PHB substratum. Postlarvae provided with PVC substratum have a median weight of 502.2 mg with a minimum-maximum weight of 31.6-1 407.3 mg while postlarvae provided with PHB substratum have a median weight of 495.2 mg with a minimum-maximum value of 192.6-903.40 mg.

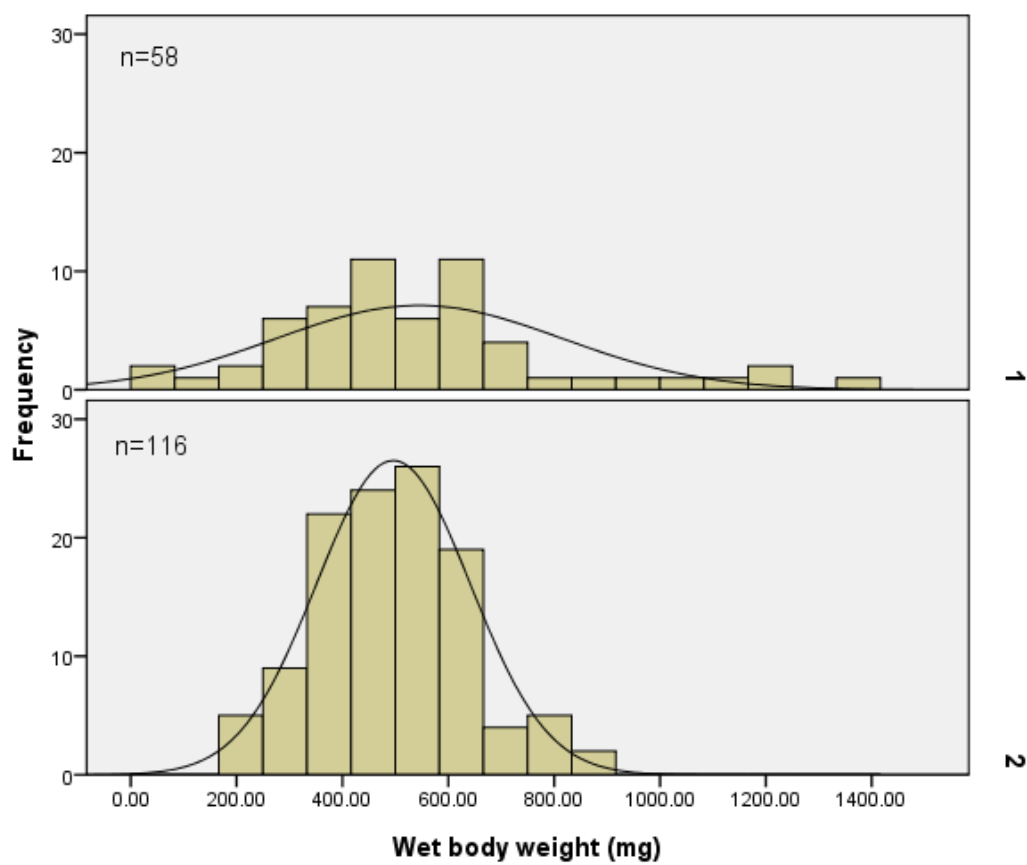


Figure 5.1 Weight frequency distribution of all surviving postlarval tiger shrimp provided either PVC (1) or PHB (2) substratum in the culture tank.

5.3.1.2 Stress test

The proportion of postlarvae surviving after exposure to a lethal concentration of ammonium chloride is shown in Figure 5.2. The final mean proportion of surviving postlarvae after 99 h were 0.0 ± 0.0 % and 0.1 ± 0.0 % in treatment provided with the PVC and PHB substrata, respectively.

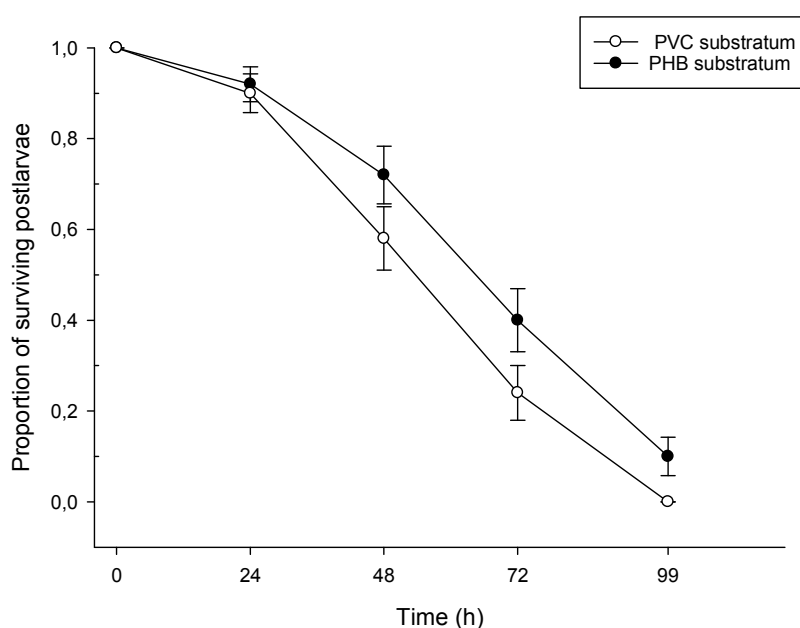


Figure 5.2 Proportion of surviving postlarval tiger shrimp cultured with either PVC or PHB substratum and subsequently exposed for 99 h to a lethal dose of ammonium chloride at 143 mg L^{-1} with a mean pH of 8.2. Values represent means \pm SEM ($n = 5$).

Overall, the proportion of surviving postlarvae between treatments did not significantly differ in any time point although there was a trend of higher survival in postlarvae provided PHB substratum as compared to PVC substratum.

5.3.1.3 *Vibrio* challenge test

Figure 5.3 shows the proportion of postlarval tiger shrimp surviving after 24 h exposure to a sub-lethal ammonium chloride dose followed by bath challenge with the pathogen *V. campbellii* LMG21363.

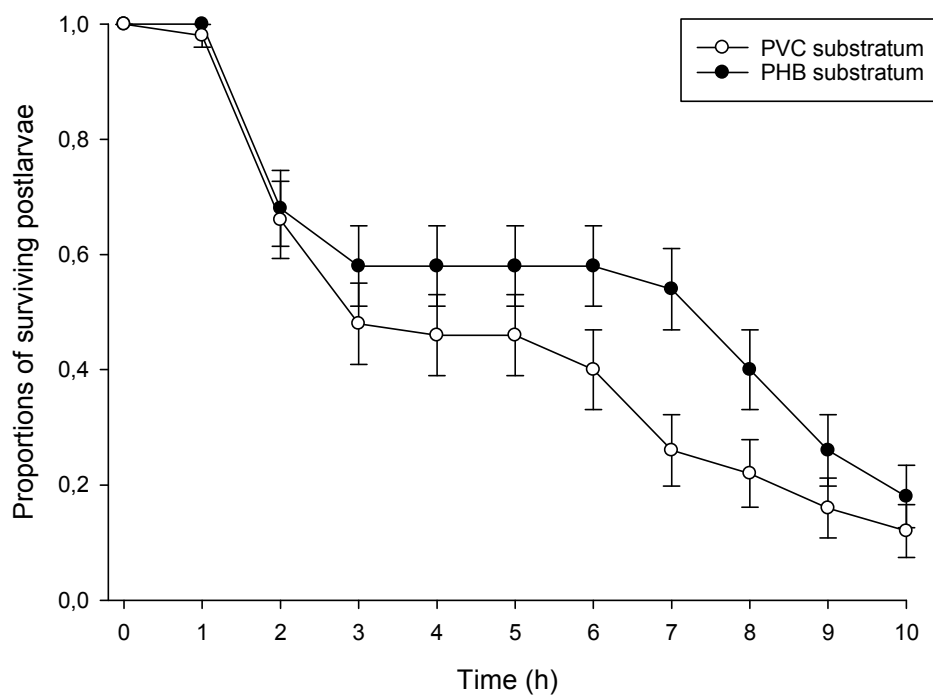


Figure 5.3 Proportion of surviving postlarval tiger shrimp cultured with either PVC or PHB substratum followed by exposure to a sub-lethal dose of ammonium chloride for 24 h and subsequent challenge with pathogenic *V. campbellii* LMG 21363. Values represent means \pm SEM ($n = 5$).

The final mean proportion of surviving postlarvae at day 10 post-challenge were 0.1 ± 0.0 % and 0.2 ± 0.1 % in treatment provided with the PVC and PHB substrata, respectively. Overall, the proportion of surviving postlarvae between treatments did not significantly

differ in any time point although there was a trend of higher survival in postlarvae provided PHB substratum as compared to PVC substratum.

5.3.1.4 Substratum visit test

Results on the substratum visit are shown in Figure 5.4. The visit (%) of postlarvae

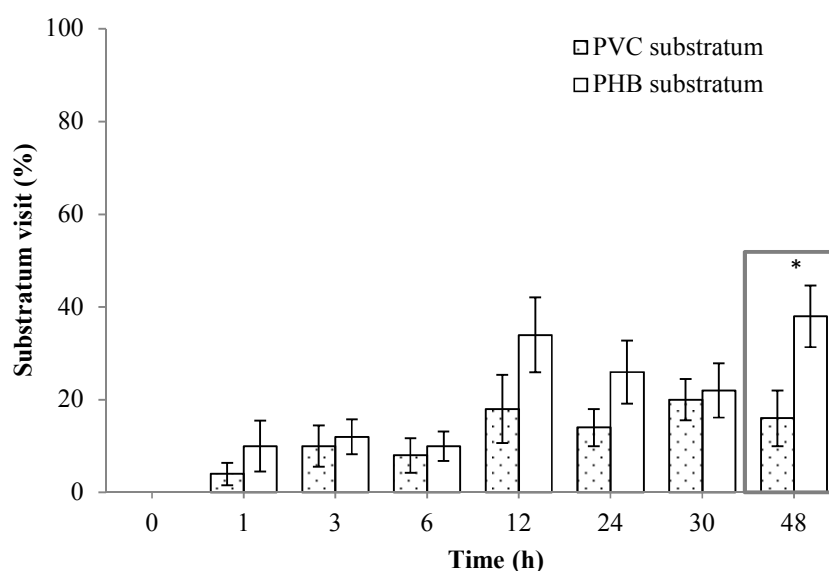


Figure 5.4 Substratum visit (%) by postlarval tiger shrimp on PVC and PHB substrata. Data are represented as means \pm SEM ($n = 5$). Asterisk indicates significant differences between treatments within a time point as indicated with a box ($P \leq 0.05$).

either to PVC or PHB substratum was low during the first 6 h but this increased from 12 until 48 h. At 48 h, a significantly higher visit of postlarvae was observed on PHB substratum (38 ± 6.6 %) as compared to PVC substratum (16 ± 6.0 %). Overall, there was

a trend of higher visit in any time point by postlarvae on PHB substratum over PVC substratum.

5.3.1.5 Lipid and fatty acid composition of the tiger shrimp postlarvae

The whole-body lipid contents (% on DW) and contents of four essential FAs of the postlarval tiger shrimp provided either PVC or PHB substratum are shown in Table 5.3. The whole-body lipid contents did not differ significantly in postlarvae provided PVC or the PHB substratum ($F = 1.307$, $P = 0.307$). The whole-body contents expressed on DW and on total lipid of two essential PUFAs linoleic acid (LOA; 18:2 (n-6)) and linolenic acid (LNA; 18:3 (n-3)) and the essential highly unsaturated FA (HUFA) docosahexaenoic acid (DHA; 22:6 (n-3)) were also not significantly different in postlarvae provided either PVC or PHB substratum. The whole-body contents of the n-3 essential HUFA EPA did not change although content expressed on total lipid was significantly higher in postlarvae provided PHB substratum ($84.4 \pm 1.1 \text{ mg g}^{-1} \text{ lipid}$) as compared to postlarvae provided PVC substratum ($74.5 \pm 3.3 \text{ mg g}^{-1} \text{ lipid}$). Similarly, the whole-body contents of the total n-3 essential FAs did not change but these were significantly higher in postlarvae provided PHB substratum ($164.8 \pm 2.2 \text{ mg g}^{-1} \text{ lipid}$) as compared to postlarvae provided PVC substratum ($151.5 \pm 4.4 \text{ mg g}^{-1} \text{ lipid}$), when expressed on lipid content. Contents of the total n-6 essential FAs and the ratio of n-3/n-6 essential FAs on whole body and on total lipids were not significantly different in postlarvae provided either PVC or PHB substratum.

Table 5.3 Whole-body lipid contents (% on DW) and contents of four essential FAs expressed either on dry weight (mg g⁻¹ DW) or on total lipid (mg g⁻¹ lipid; in parentheses) of postlarval tiger shrimp cultured for 61 days in the presence of either PVC substratum or PHB substratum. Values are means \pm SEM. Different superscript letters in a column indicate significant differences ($P \leq 0.05$; P -values in **bold**).

Substratum types	Total lipids	Essential FAs				Total essential n-6 FAs	Total essential n-3 FAs	n-3/n-6 ratio
		<i>LOA</i> (18:2 (n-6))	<i>LNA</i> (18:3 (n-3))	<i>EPA</i> (20:5 (n-3))	<i>DHA</i> (22:6 (n-3))			
PVC	5.44 \pm 0.20 ^a	2.62 \pm 0.08 ^a (48.38 \pm 1.71 ^a)	0.20 \pm 0.01 ^a (3.60 \pm 0.12 ^a)	4.03 \pm 0.08 ^a (74.46 \pm 3.28 ^b)	3.99 \pm 0.08 ^a (73.44 \pm 1.48 ^a)	2.62 \pm 0.08 ^a (48.38 \pm 1.71 ^a)	8.21 \pm 0.12 ^a (151.5 0 \pm 4.73 ^b)	3.14 \pm 0.06 ^a (3.14 \pm 0.06 ^a)
PHB	5.01 \pm 0.15 ^a	2.58 \pm 0.17 ^a (51.30 \pm 2.06 ^a)	0.20 \pm 0.01 ^a (4.01 \pm 0.17 ^a)	4.23 \pm 0.13 ^a (84.40 \pm 1.07 ^a)	3.83 \pm 0.08 ^a (76.48 \pm 1.34 ^a)	2.58 \pm 0.17 ^a (51.30 \pm 2.06 ^a)	8.26 \pm 0.22 ^a (164.88 \pm 2.15 ^a)	3.23 \pm 0.13 ^a (3.23 \pm 0.13 ^a)
<i>P</i>-value	0.307	0.819 (0.306)	0.673 (0.081)	0.226 (0.020)	0.213 (0.166)	0.819 (0.306)	0.849 (0.033)	0.517 (0.517)

5.3.2 Experiment 2: Use of PHB substratum for whiteleg shrimp postlarvae

5.3.2.1 Measured parameters

The survival and growth of postlarval whiteleg shrimp at the end of the experimental trial are shown in Table 5.4. Significantly higher survival was noted for postlarvae provided PHB substratum as compared to postlarvae provided PVC substratum ($t(12) = -2.505$, $P = 0.028$). Similarly, the final mean wet BW ($t(12) = -2.139$, $P = 0.054$) and mean daily growth ($t(12) = -2.139$, $P = 0.054$) were significantly higher in postlarvae provided PHB substratum as compared to postlarvae provided PVC substratum.

Table 5.4 Survival (%) and growth of postlarval whiteleg shrimp provided either PVC or PHB substratum in the culture tank. Values represent means \pm SEM ($n = 7$). Different letters within a row indicate significant differences ($P \leq 0.05$; P -value in **bold**).

Measured parameters	Substratum types		P -value
	PVC	PHB	
Survival (%)	73.9 \pm 3.0 ^b	83.6 \pm 2.4 ^a	0.028
Mean wet body weight (mg)	893.4 \pm 29.9 ^b	1008.2 \pm 44.5 ^a	0.054
Mean daily growth (mg day ⁻¹)	14.3 \pm 0.5 ^b	16.4 \pm 0.8 ^a	0.054

Figure 5.5 shows the weight frequency distribution of all the surviving postlarvae sampled in treatment provided either PVC or the PHB substratum. The postlarvae provided PVC substratum have a median weight of 884.8 mg with a minimum-maximum weight of 217.5-1 875.9 mg while postlarvae provided PHB substratum have a median weight of 938.6 mg with a minimum-maximum value of 488.2-2 012.5 mg.

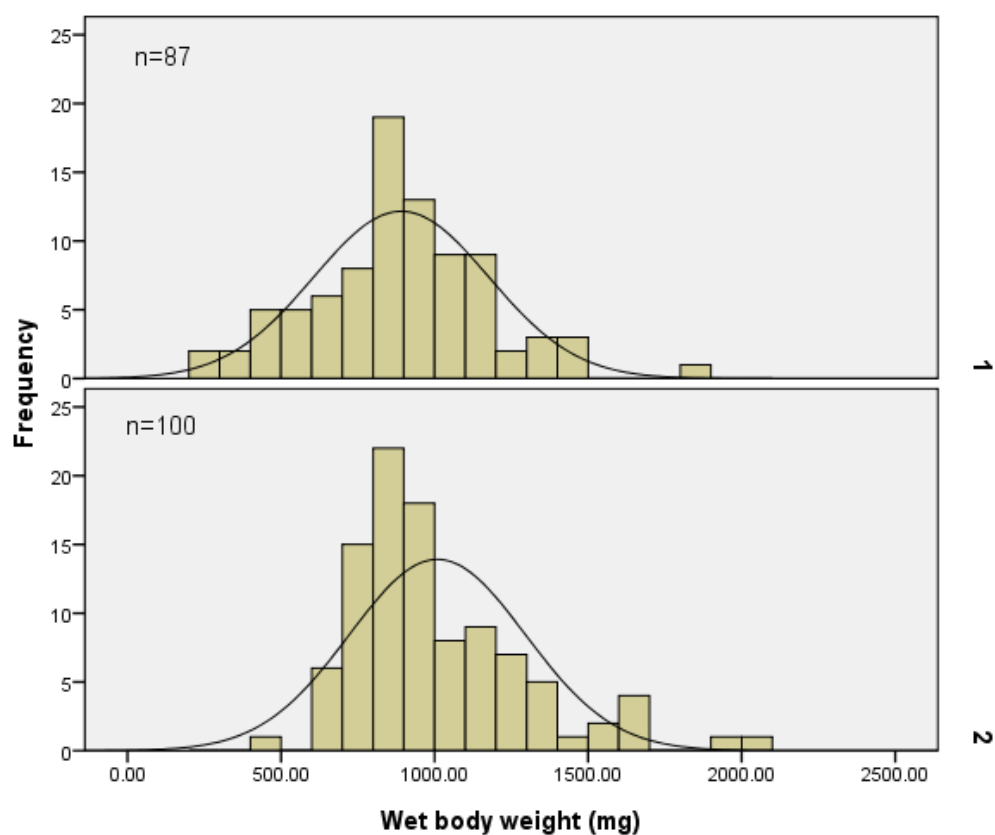


Figure 5.5 Weight frequency distribution of all surviving postlarval whiteleg shrimp provided either PVC (1) or PHB (2) substratum in the culture tank.

The faecal matter collected from postlarvae provided PVC substratum was mainly brown in coloration which closely resembled the color of the diet whereas a combination of brown and white faecal strips was observed for postlarvae provided PHB substratum (Figure 5.6).

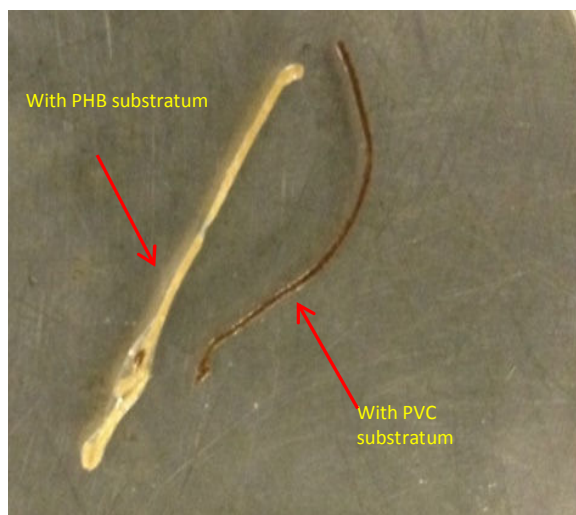
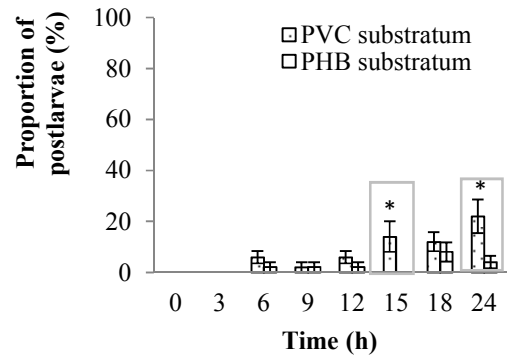
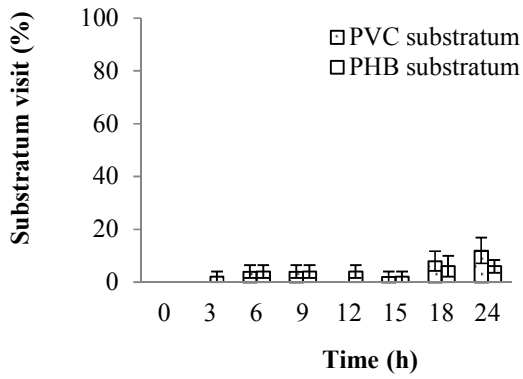


Figure 5.6 Faecal matter collected from postlarval whiteleg shrimp cultured in the presence of either PVC substratum or PHB substratum.

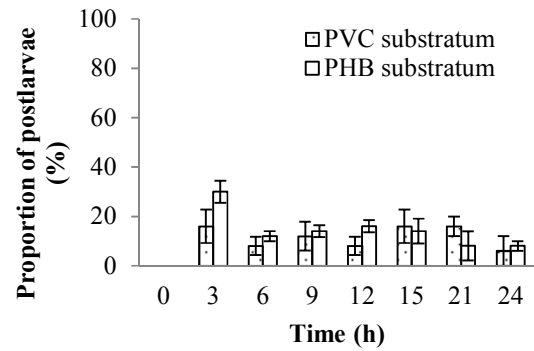
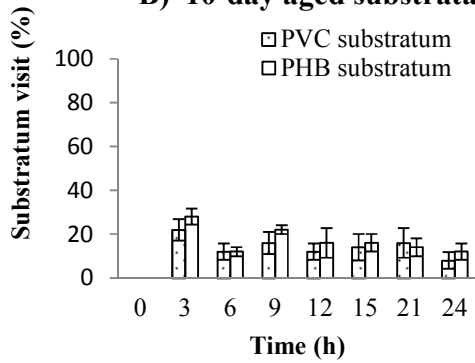
5.3.2.2 *Substratum visit and preference test*

The visit and preference by postlarval whiteleg shrimp on non-aged (test 1), 10-day aged (test 2) and 70-day aged (test 3) substrata are presented in Figure 5.7. The visit of postlarvae for non-aged PVC and PHB substrata was low and did not significantly differ at any time point (panel A, left). According to the substratum preference test, a trend of higher postlarval preference was observed for non-aged PVC substratum over the non-aged PHB substratum and this was significantly higher at time points 15 and 24 h (panel A, right). For 10 day-aged substratum test, there was a trend of higher visit by postlarvae on PHB substratum over PVC substratum (panel B, left).

A) Non-aged substrata



B) 10-day aged substrata



C) 70-day aged substrata

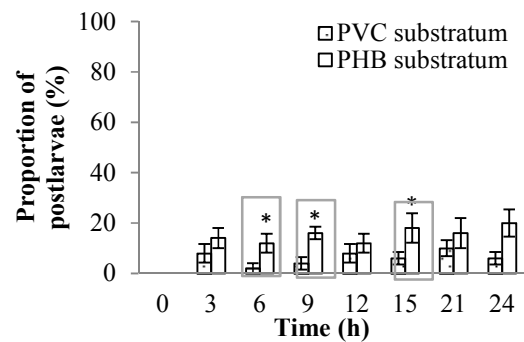
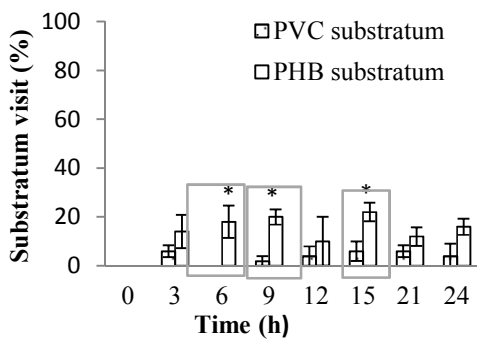


Figure 5.7 Visit and preference by postlarval whiteleg shrimp on PVC and PHB substrata. Values are represented as means \pm SEM ($n = 5$). Asterisk indicates significant differences between treatments within a time point as indicated with a box ($P \leq 0.05$).

According to the substratum preference test, a trend of higher postlarval preference (except for 15 and 21 h) was observed on 10-day aged PHB substratum over 10-day aged PVC substratum (panel B, right). For the 70-day aged substratum test, there was a trend of higher visit on 70-day aged PHB substratum over 70-day aged PVC substratum and this was significantly higher at time points 6, 9 and 15 h (panel C, left). According to the substratum preference test, a trend of higher postlarval preference was observed on 70-day aged PHB substratum over the 70-day aged PVC substratum and this was significantly higher at time points 6, 9 and 15 h.

5.3.3 Experiment 3: Effect of PHB substratum on water quality

The changes in the levels of TAN, NO_2^- -N, NO_3^- -N and TIN in the tanks without substratum or provided with substratum consisting of either PVC or PHB in the presence of an initial concentration of 1.75 mg L^{-1} TAN are presented in Figure 5.8. Results from a one-way ANOVA showed significantly lowest TAN level at all time points (except for 72 h) in the PHB substratum treatment as compared to either control or the PVC substratum treatment (panel A, left). For the NO_2^- -N, there was a trend of highest NO_2^- -N level in the PHB substratum treatment and this was significantly highest in time points 12, 18, 24 and 48 h as compared to either control or the PVC substratum treatment (panel B, right). For the NO_3^- -N, levels were not significantly different among treatments in any time point (panel C, left).

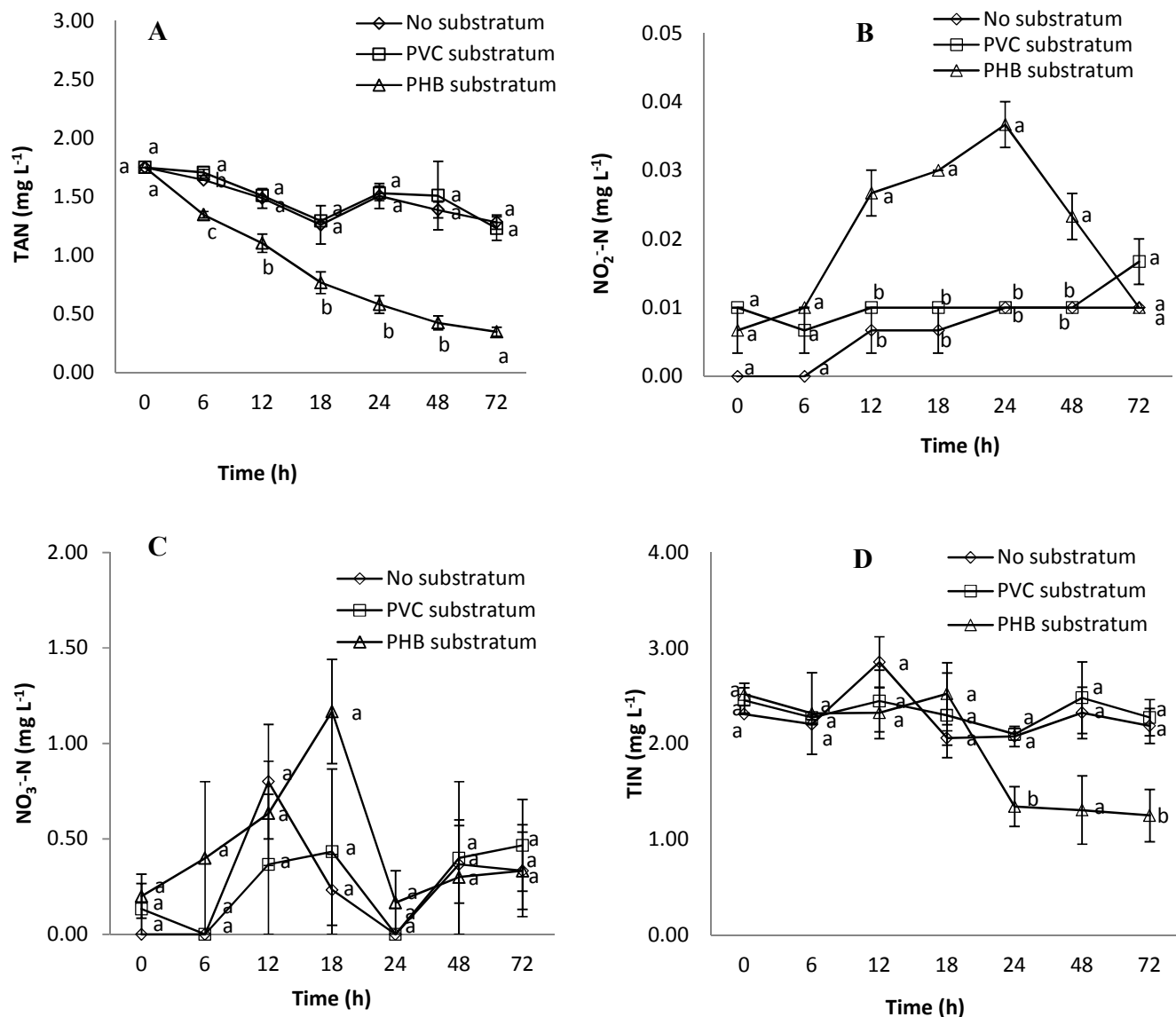


Figure 5.8 Changes in the levels of TAN ($\text{NH}_3\text{-N} + \text{NH}_4^+\text{-N}$; panel A), nitrite-nitrogen ($\text{NO}_2\text{-N}$; panel B), nitrate-nitrogen ($\text{NO}_3\text{-N}$; panel C) and their sum (TIN; panel D) in tanks provided with no substratum (control), PVC substratum or PHB substratum in the presence of an initial concentration of 1.75 mg L^{-1} TAN. Data are means \pm SEM ($n = 3$). Different letters indicate significant differences among treatments at a specific time point ($P \leq 0.05$).

Overall, the level of TIN at 24 and 72 h were significantly lowest in PHB treatment as compared to either the control or the PVC substratum treatment (panel D, right).

The changes in the levels of the TAN, NO_2^- -N, NO_3^- -N and the sum of these parameters (TIN) in the tanks without substratum or the tanks provided with substratum consisting of either PVC or PHB in the presence of whiteleg shrimp postlarvae at a density of 1 individual per L^{-1} are presented in Figure 5.9. Results from a one-way ANOVA showed a non significantly different TAN level among treatments at any time point (panel A, left). For the NO_2^- -N, there was a trend of highest NO_2^- -N level at all time points in the PHB substratum treatment as compared to either the control or the PVC substratum treatment (panel B, right). For the NO_3^- -N level, there were no significant differences among treatments at any time point (panel C, left). Overall, the level of TIN did not significantly differ among treatments at any time point (panel D, right).

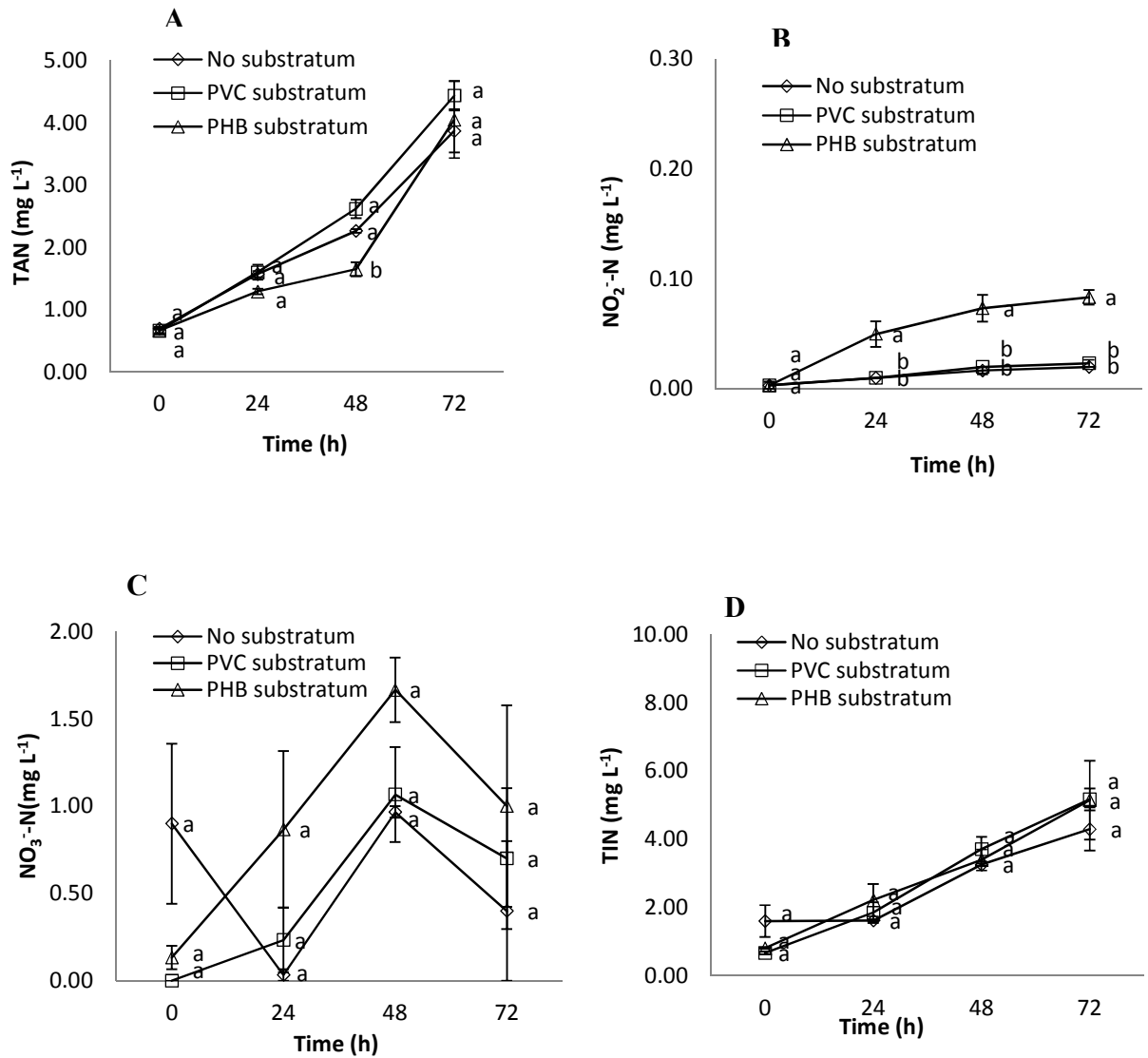


Figure 5.9 Changes in the levels of TAN ($\text{NH}_3\text{-N} + \text{NH}_4^+\text{-N}$; panel A), nitrite-nitrogen ($\text{NO}_2\text{-N}$; panel B), nitrate-nitrogen ($\text{NO}_3\text{-N}$; panel C) and their sum (TIN; panel D) in tanks provided no substratum (control), PVC substratum or PHB substratum in the presence of whiteleg shrimp postlarvae at a density of 1 individual per L^{-1} . Data are means \pm SEM ($n = 3$). Different letters indicate significant differences among treatments at a specific time point ($P \leq 0.05$).

5.4 Discussion

In this study, the use of PHB as biodegradable substratum in penaeid shrimp culture was investigated. The culturing of tiger shrimp postlarvae in the presence of PHB substratum did not result in growth promoting effects as seen by the comparable mean weight in postlarvae provided with PVC and PHB substrata. Whiteleg shrimp postlarvae, however, showed a significantly higher final mean weight in the presence of PHB substratum as compared to PVC substratum. Our data on mean daily growth also showed significantly higher growth rate in whiteleg shrimp postlarvae provided PHB substratum as compared to postlarvae provided PVC substratum. These results may indicate species-specific trait. Studies conducted on other crustacean species have also indicated that the growth-promoting effects of PHB could be species dependent. For instance, Sui *et al.* (2012) and Nhan *et al.* (2010) have shown that larval development of Chinese mitten crab *Eriocheir sinensis* and giant freshwater prawn *M. rosenbergii*, respectively, were significantly improved when given PHB-enriched *Artemia*. However, the findings obtained in Chapter 2 using *P. monodon* (early mysis stage until PL21-22) showed comparable final mean weight for postlarvae fed non-enriched *Artemia* and postlarvae fed PHB-enriched *Artemia*. The different types of PHB substratum used in the present study may also have contributed to this difference. The PHB substratum (PHB type P209) used in culturing postlarval whiteleg shrimp was thinner as compared to PHB substratum (PHB type DP9002) used in culturing postlarval tiger shrimp and this may have resulted in better ingestion and digestion of PHB fraction. From the color of the faecal pellets, it is evident that the shrimp also consumed fractions of the PHB substratum which potentially

allowed PHB to work in a similar way as has been described as enrichment product or feed additive. It could also be, however, that the biofilm assemblage in PHB substratum consisting of PHB type P209 may have differed from the biofilm assemblage in PHB substratum consisting of PHB type DP9002. Considering the weight frequency distribution of either tiger shrimp or whiteleg shrimp postlarvae, the provision of PHB substratum seemed to result in a homogenous size as compared to PVC substratum. This effect was more pronounced in postlarval tiger shrimp than in postlarval whiteleg shrimp.

It was also shown that the use of substratum consisting of PHB-based plastic improved the survival of either postlarval tiger shrimp or whiteleg shrimp. The survival enhancing effects of PHB incorporated in the feed for crustaceans have earlier been reported in literature. Sui *et al.* (2012) and Nhan *et al.* (2010) observed an increase in survival for larvae of Chinese mitten crab *E. sinensis* and the giant freshwater prawn *M. rosenbergii*, respectively, when fed crystalline PHB enriched in *Artemia* nauplii. The use of PHB in amorphous form either as feed additive for postlarval *P. monodon* (Laranja *et al.* 2014) or larval *M. rosenbergii* (Thai *et al.* 2014) also resulted in a significant increase in its survival. The survival enhancing effects of PHB have earlier been attributed to its delivery of energy following ingestion and gastrointestinal degradation (De Schryver *et al.* 2010a; Defoirdt *et al.* 2007b). As mentioned earlier, shrimp also consumed fractions of the PHB substratum which potentially allowed PHB to work in a similar way as has been described before. The improved survival may also be related to the formation of biofilm on the surface of the PHB substratum. According to Otoshi *et al.* (2006), substratum provides additional surface area upon which shrimp grazes making the biofilm

act as a supplementary source of feed. Although biofilms also established on the surface of the PVC substratum, the quality of the biofilm assemblages developing on PVC and PHB substrata may have been different. Reports from literature have indicated that biofilm communities and/or bacterial densities may differ in response to substratum type. For instance, Faimali *et al.* (2004) showed different biofilm assemblages in substrata consisting of marble, quartz, glass and cembonit. These authors have also demonstrated a shift of biofilm assemblage in function of substratum age (5, 10 and 20-day aged). Likewise, Hung *et al.* (2008) showed different diatom communities between substrata consisting of glass and polystyrene. Chung *et al.* (2010) found higher bacterial densities in substratum consisting of granite rock panels as compared to substratum consisting of polystyrene petri dish. These differences may be influenced by chemical and physical cues of the surface (Renner & Weibel 2011). Niquette *et al.* (2000) have indicated that surface's porosity and roughness are important factors influencing bacterial density. In the present study, however, tests were carried out using sandpaper-roughen PVC and PHB substrata and thus surface roughness can be ruled out. The surface chemistry, i.e. the chemical properties of the materials, has also been shown to directly affect microbial attachment and survival (Whitehead & Verran 2009). For the PHB substratum, reports in literature have mentioned that various microorganisms are capable of excreting extracellular PHB depolymerases that hydrolyse PHB into water-soluble oligomers and monomers and subsequently utilise these resulting products as nutrients within the cells (Sudesh *et al.* 2000). Additionally, Rostkowski *et al.* (2012) stated that PHB-based bioplastic is completely biodegraded into water and carbon dioxide (and methane under

anaerobic conditions) by bacteria in natural environments, including water, soil and compost. The degraded PHB substratum seen in the present study may indicate utilization by microorganisms as previously described. Several studies have also demonstrated that the addition of artificial substratum in penaeid shrimp culture is an ideal method in increasing survival by minimizing the negative effects of overcrowding (Ballester *et al.* 2007; Bratvold & Browdy 2001). In the present study, however, the PHB substratum used in Experiment 1 already showed signs of degradation resulting from aging prior to the onset of the experimental trial. In addition, the stocking density of the postlarvae in the PVC and PHB treatments was low (1 individual L⁻¹). Therefore, it is speculated that the importance of PHB substratum for tiger shrimp postlarvae is mainly related to the ingestion of the PHB itself and the presence of additional food provided by the biofilm assemblages. It should be mentioned, however, that the PHB substratum used in Experiment 2 were intact and retained its form after aging. Although PHB bioplastic in Experiment 1 has thicker walls (0.05 cm) as compared to PHB bioplastic used in Experiment 2 (0.03 cm), the latter was more flexible and does not easily break when rolled as compared to the former which is stiff and brittle. One possible reason for this difference could be the type or proportion of plasticizer being used.

The trend of higher visit by postlarval tiger shrimp on 76-day aged PHB substratum in any time point also suggests the importance of PHB substratum as grazing area rather than as shelter. This was also the case for the postlarval whiteleg shrimp showing higher visit and preference for 10-day and 70-day aged PHB substratum over 10-day and 70-day aged PVC substratum.

The use of PHB substratum seemed to enhance the robustness and resistance of postlarval tiger shrimp against adverse environmental conditions and pathogenic pressure as seen by the trend of higher survival for postlarvae provided PHB substratum as opposed to PVC substratum, although the differences were not significant. The robustness and resistance enhancing effects of PHB-accumulating bacteria in postlarval tiger shrimp was demonstrated by Laranja *et al.* (2014). As the PHB in the present study was not administered via the feed, the robustness and enhancement effects must have been due to the presence of PHB as a substrate in the water. The same aspects as mentioned above – ingestion of PHB substratum or influence of specific biofilm assemblage on the surface – may have contributed in this aspect. The provision of PHB substratum also seemed to influence the essential n-3 HUFA EPA by increasing its level. This could be beneficial as essential FAs such as EPA are important for penaeid shrimp as they play a role in the enhancement of immune parameters and resistance to bacterial and viral pathogens (Parrish 2009).

The effect of PHB substratum on water quality was seen in the present study. Provision of PHB substratum seemed to effectively decrease the level of TAN as seen in test 1, however, this was not the case for test 2. The reason for this may be that the available biofilm surface was likely not enough for the amount of nitrogen released by the shrimp. In addition, the degrading nature of PHB resulting in surface loss may not be sufficient for biofilm formation. Ebeling *et al.* (2006) have mentioned that the removal of TAN in aquaculture systems can be achieved through 3 pathways: photoautotrophic removal by algae, autotrophic bacterial conversion of ammonium–nitrogen to nitrate–

nitrogen and heterotrophic bacterial conversion of ammonia–nitrogen directly to microbial biomass. In a study conducted by Thompson *et al.* (2002), the significant reduction of ammonium and the consistently low levels of nitrite and nitrate from the culture water of the pink shrimp *Farfantepenaeus paulensis* provided with mature biofilm were attributed to the pennate diatoms and filamentous cyanobacteria that mainly absorbed ammonium and use this element to produce new biomass. In the present study, however, we have seen a trend of elevated levels of nitrite-nitrogen and nitrate-nitrogen during the experimental trial and this was consistent in both PVC and PHB substrata. From this, it is initially thought that nitrifying bacteria specifically the ammonia-oxidizing bacteria (AOB) such as *Nitrosomonas*, *Nitrospira* and *Nitrosococcus* (Burrell *et al.* 2001) could be the main actors responsible in decreasing the TAN in test 1. However, from our calculations on nitrogen mass balance, we could see no strong evidence that this is indeed the case. For instance, the TAN level in treatment consisting of PHB substratum reduced by 0.34 mg L⁻¹ from 12 h to 18 h while the sum of nitrite- and nitrate-nitrogen increased by 0.54 mg L⁻¹, respectively, indicating excessive production of either nitrite-nitrogen or nitrate-nitrogen. Based from this calculation, it is hypothesized that the TAN removal seen in treatment consisting of PHB substratum was not accomplished merely by nitrification and therefore, further study is warranted to determine which of the abovementioned processes is responsible for improving the water quality by removal of TAN as seen in test 1. Although there was an elevation of nitrite-nitrogen and nitrate-nitrogen in both tests, levels are within the recommended safe levels for penaeid shrimp to be at < 0.2-0.25 mg L⁻¹ (Wickins & Lee 1992) and 20 mg L⁻¹ (Camargo *et al.* 2005).

In conclusion, this study is the first to demonstrate the potential beneficial effects of PHB-based bioplastic as artificial substratum in penaeid culture. The provision of PHB substratum resulted in enhanced survival and overall performance of penaeid shrimp. PHB substratum also serves as an important food source either by directly ingesting it or by grazing on the biofilm assemblages. Finally, PHB substratum contributed to the improvement of water quality by decreasing the levels of total ammonia-nitrogen.

Acknowledgement

The authors are grateful to the staff of Metabolix GmbH (Köln, Germany) and U.J. Hanggi (Biomer company, Krailling, Germany) for kindly providing PHB type DP9002 and PHB type P209, respectively.

*“Hey, George, the ocean called and
they're running out of shrimp”*

-The Comeback (Seinfeld)

Chapter 6

Conclusions and future perspectives

General discussion and conclusions

6.1 Introduction

The application of PHB has been explored in various aquatic farmed species and it was shown to promote growth (Nhan *et al.* 2010; Sui *et al.* 2012), enhance resistance against pathogens (Defoirdt *et al.* 2007b; Sui *et al.* 2012, Suguna *et al.* 2014, Halet *et al.* 2007, Laranja *et al.* 2014; Thai *et al.* 2014) and serve as an energy source (De Schryver *et al.* 2010a, Defoirdt *et al.* 2007b). Recently, PHB has been shown to affect lipid metabolism in fish (Najdegerami *et al.* 2015; Situmorang 2015) and it is distributed in different organs following digestion and absorption (Situmorang 2015). Despite the current reports on the beneficial effects of PHB in aquatic farmed species, its actual mechanisms of actions in aquatic farmed species remained largely unexplored. In addition, there are also very few attempts made to explore the application of PHB in penaeid shrimp being the most important internationally traded fishery commodity in terms of value and the most valuable fishery export for many tropical developing countries.

6.2 Main accomplishments

This research has successfully demonstrated the application of PHB in penaeid shrimp and its effects are highlighted below:

- This work is the first to demonstrate that crystalline PHB (98% poly- β -hydroxybutyrate - 2% poly- β -hydroxyvalerate) supplied via *Artemia* in a short term-enrichment strategy resulted in enhanced survival in early developmental stage of

tiger shrimp *Penaeus monodon*. Although PHB could not be used to stimulate growth, it was found to increase the survival after exposure to adverse environmental condition and enhanced the resistance against pathogenic bacteria. Co-supplying PHB with HUFA (highly unsaturated fatty acid)-rich lipid emulsion employing a short-term enrichment strategy nullified these effects (Chapter 2).

- The effects of supplementing various forms of PHB on the whole body lipid contents and contents of four fatty acids considered essential to penaeid shrimp using *Artemia* sp. as model species was demonstrated for the first time. Supplying PHB either in crystalline form or amorphous form in lyophilized cells of *Ralstonia eutropha* DSM545 with or without the addition of PHB-degrading bacterium *Comamonas testosteroni* resulted in a lipid-saving effects indicating that PHB may act as an alternative energy source in crustaceans as previously reported in other aquaculture species. Supplying amorphous PHB only or crystalline form in combination with PHB-degrading bacterium *C. testosteroni* significantly increased the contents of the two essential n-6 polyunsaturated FAs (PUFAs) linoleic acid (LOA; 18:2 (n-6)) and linolenic acid (LNA; 18:3 (n-3)). This strategy, however, did not influence the contents of the essential n-3 HUFAs eicosapentanoic acid (EPA; 20:5 (n-3)) and docosahexaenoic acid (DHA; 22:6 (n-3)). PHB co-supplied to HUFA-rich lipid emulsion in a long-term enrichment strategy did not significantly affect the nutritional quality of the lipid-supplied *Artemia* nauplii (Chapter 3).
- The application of the ^{13}C stable isotope analysis has demonstrated for the first time the assimilation of ^{13}C -labeled *R. eutropha* DSM545 containing 75 % PHB on DW in

two crustacean species: postlarval whiteleg shrimp *Litopenaeus vannamei* and *Artemia* sp. nauplii. Supplementation of 2 % ^{13}C -labeled *R. eutropha* DSM545 in the diet of postlarvae did not result in massive ^{13}C transfer from the diet to the different long chain fatty acids (LCFAs) in various lipid fractions of the postlarvae indicating that PHB offered in natural matrix (e.g. contained in a bacterial carrier) may not be used as a building block but probably rather as energy source. Diet composition did not influence ^{13}C enrichment in both crustacean species which might indicate that assimilation of PHB offered in natural matrix is not interfered by diet composition. There was also an indication that PHB offered in its natural matrix is rapidly assimilated in the tissues of crustaceans, however, results on fatty acid specific analysis have indicated that PHB is not assimilated in its fatty acid components, specifically LCFAs (Chapter 4).

- This work has also demonstrated, for the first time, that biodegradable plastic made up out of PHB-based bioplastic provides a good alternative over conventional substratum material consisting of polyvinylchloride (PVC) pipes as artificial substratum in penaeid shrimp. Its application has resulted in improved survival and overall performance of either postlarval tiger shrimp or whiteleg shrimp. Further, it was demonstrated that PHB substratum can effectively decrease the levels of total ammonia-nitrogen indicating its potential as agent for water quality management (Chapter 5).

6.3 Disease management in aquaculture

Diseases represent one of the major risks to aquaculture operations (Kobayashi & Melkonyan 2011) as it can reduce economic value by decreasing meat quality, increase marginal costs of harvest and processing and diminish biological productivity (Lafferty *et al.* 2015). Arrays of stress factors such as poor water quality and parasite load are often associated with outbreaks of disease (Austin & Austin 1999). In Figure 6.1, the interactive factors influencing disease in aquaculture is outlined.

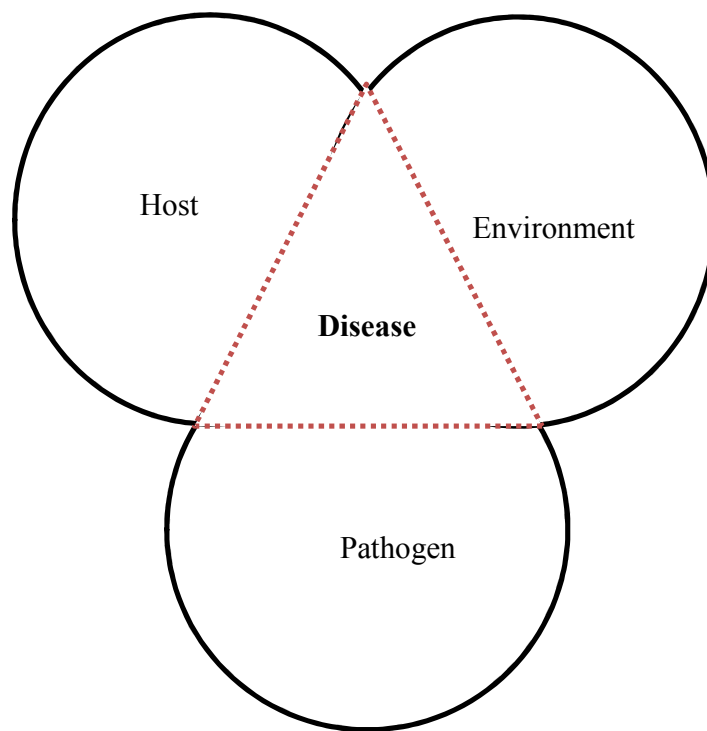


Figure 6.1 Interactive factors influencing disease in aquaculture (redrawn from Nemutanzhela *et al.* 2014).

Disease is the result of a disturbance in the delicate balance between host, pathogen and environment. Strategies to prevent and control bacterial disease in aquaculture should therefore ideally take into account the different aspects of the pathogen-host-environment continuum (Defoirdt *et al.* 2011).

6.4 The mode of action of poly- β -hydroxybutyrate: Host

6.4.1. Survival-enhancing effects

In this work, the survival enhancing effects of PHB in penaeid shrimp were demonstrated in two different cases: supplying PHB via *Artemia* nauplii irrespective of lipid emulsion (Chapter 2) and provision of PHB biodegradable plastic as artificial substratum during culture (Chapter 5).

The survival-enhancing effects of PHB either as enrichment product or feed additive in fish and other crustacean species are documented and one common understanding regarding this effect is the capacity of PHB to act as energy source as mentioned by De Schryver *et al.* (2010a). In the present work, there have been several instances that demonstrated the potential of PHB as energy source of penaeid shrimp. For instance, the whole-body lipid contents of starved *Artemia* nauplii were significantly increased when PHB either in crystalline or amorphous form irrespective of PHB-degrading bacterium *C. testosteroni* (Chapter 3). In postlarval whiteleg shrimp *L. vannamei*, supplementing 2 % ^{13}C -labeled *R. eutropha* DSM545 containing 75 % PHB on DW on its diet did not result in massive transfer of ^{13}C in various lipid fractions indicating that PHB offered in natural matrix (e.g. contained in bacterial cell) could not be used as

building block but as energy source (Chapter 4). The capacity of PHB and/or its degradation product β -HB to act as alternative source of energy could spare endogenous lipid reserves for use in other energy-demanding processes such as molting, reproduction and handling of pathogens, thereby increasing their chance of survival during these stressful episodes (Raviv *et al.* 2008).

The survival enhancing effects of PHB offered as artificial substratum during the culture of either postlarval tiger shrimp or whiteleg shrimp is also reported. Here, it is hypothesized that the consumption of PHB fractions could have potentially allowed the PHB to work in a similar way when offered as enrichment product or feed additive. The role of biofilm could have also contributed to the survival enhancing effects of PHB substratum. In general, artificial substratum is regarded as an important source of natural food through the biofilm formed on the surface (Zhang *et al.* 2010). Ballester *et al.* (2007) mentioned that biofilm formed on artificial substratum is composed of organisms that belong to the natural diet of penaeid shrimp and thus, serves an additional source of nutrition. The proximate composition of a typical biofilm varies from 23-30 % protein, 2-9 % lipid, 25-28 % nitrogen-free extract (NFE) and 16-42 % ash content (Azim & Wahab 2005; Thompson *et al.* 2002; van Dam *et al.* 2002). As penaeid shrimp typically needs 5-12 % dietary lipids on DW (Shivaram & Raj 1997), the biofilm growing on the PHB substratum can be considered sufficient to meet this requirement.

6.4.2 Growth promoting effects

Several studies have explored the growth promoting effects of PHB in various fish species including European sea bass (De Schryver *et al.* 2010a), Siberian sturgeon and Nile tilapia (Situmorang 2015) and in crustacean species including giant freshwater prawn (Nhan *et al.* 2010; Thai *et al.* 2014) and Chinese mitten crab (Sui *et al.* 2012). The growth promoting effects of PHB offered in amorphous form in postlarval tiger shrimp was also explored by Laranja *et al.* (2014). From the findings of these studies, the growth promoting effects of PHB seemed to differ between species, developmental stages and PHB dose.

In the present study, PHB supplied via *Artemia* in early developmental stage (mysis stage to early PL stage) (Chapter 2) or as artificial substratum in the culture of postlarval stage of tiger shrimp (Chapter 5) did not result in growth promotion. In the case of postlarval whiteleg shrimp, however, provision of PHB substratum as artificial substratum significantly increased its growth indicating that growth promoting effects of PHB could be species-dependent. Alternatively, the different types of PHB bioplastic used during the culture of postlarval tiger shrimp and whiteleg shrimp could have potentially contributed to this.

The significance of the two LCFAs LOA and LNA in improving growth of tiger shrimp has been described in literature (Merican & Shim 1996; Glencross *et al.* 2002) and in other penaeid shrimp species, namely *Farfantepenaeus chinensis* (Xu *et al.* 1994) and *F. indicus* (Chandge & Paulraj 1998). In the present study, supplying crystalline PHB in combination with PHB-degrading bacterium *C. testosteroni* or amorphous PHB

irrespective of *C. testosterone* resulted in significant increase of LCFAs LOA and LNA in *Artemia* nauplii. There was, however, no evaluation conducted on whether this strategy could result in parallel growth promotion in larval predator. Supplementing 2 % of ^{13}C -labeled *R. eutropha* DSM545 containing 75 % PHB on DW resulted in significant enrichment of LCFA LOA in the diet but not in the postlarval whiteleg shrimp (Chapter 4).

6.4.3 Lipid and FA profile

As mentioned earlier, lipid is an important energy store in crustaceans. Its storage and utilization is dictated on the availability of food and varying demands for growth, reproduction and maintainance (Bamstedt 1986). In fish species, PHB was found to increase the whole body lipid contents in larval Siberian sturgeon (Najdegerami *et al.* 2015) and in juvenile Nile tilapia (Situmorang 2015). The latter hypothesized that the higher total whole-body lipid contents in the PHB treatment groups may be due to a larger amount of visceral fat, which is deposited in the form of fat (triglycerides). Literature mentioning the effect of PHB supplementation on the lipid contents and FA profile of crustaceans are not available and hence, this was investigated as part of this PhD work.

The *Artemia* nauplii starved either 24 or 48 h had a lipid content of < 10 % on DW and this is lower than the reported average lipid contents in freshly-hatched *Artemia* to be at 20 % on DW (Coutteau & Mourente 1997) indicating that *Artemia* in starved state will likely use its endogenous lipid reserve for survival. According to Ritar *et al.* (2003), starvation in crustaceans at any life stages follows three distinct phases: energy-rich lipid reserves are preferentially mobilized which is typical during short-term food deprivation.

When much of the accessible lipid pool has been depleted, proteins are increasingly utilized resulting in the degradation of structures such as muscle and nervous tissue. In the final phase of starvation prior to death, structural lipids are degraded and it is in this condition that the animal has passed its point-of-no-return and do not recover after re-feeding. Based on the significantly higher lipid contents in PHB-supplied *Artemia* nauplii relative to starved *Artemia*, it can be suggested that PHB could have acted as an alternative energy source as mentioned previously, thereby sparing its endogenous lipid reserves for other important metabolic functions (Gonzalez-Felix & Perez-Velazquez 2002). In the case of postlarval tiger shrimp cultured in the presence of PHB substratum, the lipid content of approximately 5 % on DW was within the reported lipid range of biofilm to be at 2-9 % on DW (Pandey *et al.* 2014) and this did not differ from the postlarvae cultured with the conventional substratum consisting of PVC pipes. This is presumably due to the provision of diets containing high lipid (9 % on DW) which is sufficient to meet the dietary lipid requirement of most penaeid shrimp to be at 5-12 % on DW (Shivaram & Raj 1997).

The enrichment strategy with either crystalline PHB in combination with PHB-degrading bacterium *C. testosteroni* or amorphous form irrespective of *C. testosteroni* in *Artemia* nauplii resulted in a significant increase of the two essential LCFAs LOA and LNA. This finding seemed to be important as it has been demonstrated that crustaceans, especially penaeid shrimp, have a limited ability to synthesize *de novo* the n-6 LCFAs, including LOA and LNA (Kayama *et al.* 1980; Sanchez-Paz *et al.* 2006). Supplying PHB to starved *Artemia*, however, did not influence the two essential n-3 HUFAs EPA and DHA indicating that PHB supplementation is not an ideal strategy in improving contents of

these essential LCFAs. The inability of penaeid shrimp to elongate PUFAs into HUFAs including EPA and DHA (D' Abramo 1989) could also be relevant and therefore an exogenous source of HUFAs is necessary.

6.4.4 PHB assimilation in FAs

Lipids, specifically FAs and sterols, are ideal targets for compound-specific stable carbon isotope analysis as they are abundant in higher organisms (Chamberlain *et al.* 2004). It was also mentioned by Arts (1998) that FA component of lipids is predominantly dietary in origin with only as little as 2 % attributable to *de novo* synthesis and thus, predator-prey relationship can act as tracer improving the ability to visualize the pathways of carbon flow through the ecosystem.

The first evidence on PHB assimilation in aquatic farmed species was investigated by Situmorang (2015) who demonstrated the compartmental distribution of PHB in the Nile tilapia. These authors have hypothesized the route of PHB assimilation and utilization in fish species: PHB is degraded and absorbed in the intestines resulting in free FAs that are transported in the blood. The free FAs consisting of LCFAs and SCFAs are transported separately. While LCFAs are transported by the kidney and spleen which directly enter the systemic circulation, the SCFAs are transported to the liver where they are either esterified into triglycerides, converted into cholesterol or phospholipids or oxidized into ketone bodies. The latter are further circulated by the blood where they are utilized by the heart and also by the brain and muscle as their energy source. In the present study, the route of PHB assimilation and utilization as described in fish could not be made as the analysis was

conducted on whole shrimp sample. Moreover, the metabolic fate of PHB as described in fish may not be applicable in penaeid shrimp as nutrient assimilation and utilization in fish and crustaceans may vary considerably.

In the present study, the assimilation of ^{13}C -labeled *R. eutropha* DSM545 containing 75 % PHB on DW following ingestion and *in vivo* degradation in the gut of two crustacean species: postlarval whiteleg shrimp and *Artemia* nauplii was demonstrated for the first time (Chapter 4). The *R. eutropha* DSM545, during normal biomass production, produces PHB at approximately 10 % on DW (Garcia-Gonzalez, *pers. comm.*; Mozumder *et al.* 2014) indicating the ubiquitous nature of PHB (Reusch 1992). Upon nutrient deprivation and subsequent addition of ^{13}C -labeled glucose, carbon flux is shifted from biomass production to PHB synthesis and thus, approximately 65 % of the PHB is produced from the labeled glucose. From this information, it is expected that majority of the ^{13}C signal from the labeled glucose is transferred to the PHB. There is, however, no doubt that portion of ^{13}C has also ended into the structural components (e.g. FAs) of the bacteria based on the consistent enrichment of LCFAs (e.g. palmitic acid (16:0) and pentadecanoic acid (15:0)) that are commonly associated with bacteria. There was an indication that PHB is not used as building block but as energy source based on the low ^{13}C enrichment of LCFAs from various lipid fractions of postlarvae relative to the diet. There was also an indication that PHB is rapidly assimilated in the tissues of both crustacean species. This is based on the significantly higher $\delta^{13}\text{C}$ measured at either 2 or 4 h relative to 0 h. This feature seemed to be of economic relevance as PHB in penaeid culture system is more likely utilized and does not end up as waste. Johnson *et al.* (2010)

reported the minimal price of PHB to be at 4 € kg⁻¹ which is 4 times higher than that of 1 kg fish feed (De Schryver *et al.* 2010a) and therefore, effective utilization of PHB is a must. Moreover, assimilation of PHB is not interfered by either dietary composition or presence of other feed items. In the case of postlarval whiteleg shrimp, varying the fat contents in shrimp diet was considered as findings in Chapter 3 have indicated the lipid saving effects of PHB. As report in literature has mentioned that PHB and/or β -HB could act as energy source in crustaceans (Sui *et al.* 2014), it is speculated that postlarval *L. vannamei* fed a diet containing low level of dietary fats could result in lower assimilation of PHB in its tissues since PHB and/or β -HB will be inevitably used as alternative source of energy. In contrary, postlarval fed a diet containing high level of dietary fats would result in higher assimilation of PHB in its tissues as PHB and/or β -HB is not used up as energy but probably rather stored and/or incorporated in the structural components of the postlarvae. These effects, however, were not seen in the present study and thus, further studies should be conducted to better understand this aspect. The application of purified ¹³C-labeled PHB is necessary in future investigations to avoid possible interference of the structural components of PHB-accumulating bacteria as seen in the present work. Further, the effects of PHB and/or β -HB, under normal feeding conditions, should also be investigated whether this could result in ketotic acidosis as reported by Weltzien *et al.* (2000) or excreted as waste product as previously described in vertebrates (Guyton 1991). Although the findings in Chapter 4 have also indicated that PHB and/or β -HB is not assimilated in the structural components of the postlarval whiteleg shrimp, it may also be

interesting to investigate whether PHB and/or β -HB may serve as substrate for lipogenesis under normal feeding conditions.

6.5 The mode of action of poly- β -hydroxybutyrate: Pathogen

6.5.1 Effect on pathogenic bacteria

The actual evaluation on the antagonistic effects of PHB against pathogens commonly associated in penaeid shrimp culture was not evaluated in the present work, however, results on challenge tests conducted in early and postlarval stages of tiger shrimp have indicated the potential of PHB as an anti-microbial agent in penaeid shrimp culture as previously reported in other aquatic farmed species. Few studies have explored the antagonistic effects of PHB against *Vibrio* spp. In a study conducted by Nhan *et al.* (2010), they found significantly low total bacteria and *Vibrio* counts in larval giant freshwater prawn fed PHB-supplied *Artemia*.

The intestinal degradation of PHB resulting in the release of its monomer β -HB is hypothesized to be part of the PHB's action against pathogenic bacteria (Defoirdt *et al.* 2007b). One strong indication on the release of β -HB following PHB degradation is lowering of gut pH and this was observed by De Schryver *et al.* (2010b) in the gut of juvenile European seabass fed PHB-supplemented diet. The β -HB monomer acts by acidifying the cytoplasm of the pathogen. Subsequently, the pathogen redirects cellular energy to maintain homeostasis, resulting in decreased virulence (Defoirdt *et al.* 2009).

Another possible mode of action of PHB against pathogenic bacteria could be anti-adhesion as reported by Kiran *et al.* (2014). According to their findings, PHB extracted

from PHB-accumulating bacterium *Brevibacterium casei* MSI04 isolated from marine sponge *Dendrilla nigra* effectively inhibit the adhesion of biofilm-forming *Vibrio* bacteria to polystyrene surfaces and glass. This finding can be translated as a possible reduction on the capacity of *Vibrio* in colonizing farmed shrimps as well as the farm environment.

6.5.2 Effect on gut microbial community

PHB is also reported to influence the microbial community of the aquatic farmed hosts. In Siberian sturgeon fingerlings, PHB supplemented at 2 and 5 % on DW resulted in the stimulation of *Bacillus* and other bacteria belonging to the family of Ruminococcaceae (Najdegerami *et al.* 2012). These bacteria have been reported to either accumulate or degrade PHB (Liu *et al.* 2010; Laranja *et al.* 2014) and can act as probiotic that could inhibit growth of pathogenic bacteria and stimulate non-specific immune system in fish (Sakai *et al.* 1999; Rengpipat *et al.* 1998; Daniels *et al.* 2010). In the larvae of blue mussel, however, Hung *et al.* (2015) did not find bacterial stimulation in PHB-supplemented treatments. Based on these findings, the effects of PHB supplementation on gut-associated bacteria in penaeid shrimp should be investigated.

6.5.3 PHB as immunostimulant

Few studies have also indicated the potential of PHB as immunostimulant. In the crustacean model *A. franciscana*, PHB was found to confer protection to *Artemia* against *V. campbellii* by a mechanism of inducing heat shock protein (Hsp) 70. The generation of protective innate immune responses, especially the prophenoloxidase and transglutaminase, was also demonstrated (Baruah *et al.* 2015).

PHB-induced immunostimulation in fish was also reported by Suguna *et al.* (2014). Based on these findings, investigation of PHB and its monomer β -HB as immunostimulant and gene expression modulators in penaeid shrimp should also be investigated.

6.6 The mode of action of poly- β -hydroxybutyrate: Environment

6.6.1 Robustness-enhancing effects

The contribution of PHB in enhancing the robustness of early and postlarval stages of tiger shrimp was documented in Chapter 2 and 5, respectively. The ammonia stress test was employed in the present work as Colt & Armstrong (1981) mentioned that ammonia is the most common toxicant in culture systems. The robustness enhancing effects of PHB seen in the present work is related to PHB being an energy source. As it was previously pointed out by Laranja *et al.* (2014), PHB has contributed to energy delivery to postlarval giant tiger shrimp increasing their strength to resist environmental stress.

6.6.2 Water quality management

The provision of good water quality is essential to any aquaculture operations (Chien 1992). In aquaculture environment, ammonia is the major pollutant and considered to be one of the most important water quality parameters to monitor (Yue *et al.* 2010). Literature reports have mentioned of high levels of ammonia to adversely affect growth and survival (Wickins 1976; Zin & Chu 1991; Chen & Lei 1990). Excessive levels of ammonia are also reported to damage the gills and reduce the ability of haemolymph to transport oxygen while increasing oxygen consumption by tissues (Chien 1992; Racotta &

Hernández-Herrera 2000). For these reasons, steps should be taken to reduce/control ammonia production in aquaculture systems.

In Chapter 5, the results from the spiking experiment have indicated the potential of PHB substratum in decreasing total ammonia-nitrogen while maintaining nitrite-nitrogen and nitrate-nitrogen in safe levels. The elimination of nitrogenous compounds particularly ammonia in aquaculture systems is beneficial as it reduces the occurrence of pathogenic bacteria which normally occur in situations where nitrogenous compounds reach extremely high values (Austin & Austin 1999; Brock & Main 1994). The mechanism behind the decreasing levels of total ammonia-nitrogen in treatment consisting of PHB substratum is not yet known but biofilm forming in the substratum is hypothesized to be the major player. In various literature, the presence of nitrifying bacteria in the biofilm is shown to decrease the level of ammonium in the culture water (Bharti *et al.* 2013; Langis *et al.* 1988; Ramesh *et al.* 1999). Findings of Kaiser & Wheaton (1983) have also shown the elimination of ammonium with parallel augmentation of nitrite and nitrate levels as indication of the presence of nitrifying bacteria present in the biofilm. This condition was also seen in the present work in which levels of nitrite-nitrogen and nitrate-nitrogen were highest in PHB substratum as compared to treatments consisting of either no substratum or PVC substratum. Other reports have also mentioned of the role of microalgae in eliminating ammonium in the culture water to be used in the production of new biomass (Thompson *et al.* 2002). In the present work, however, the presence of microalgae in the aged substratum may be less likely since microalgae possibly thriving in the aged substratum were stripped prior to the conduct of the actual experiment by a method of

keeping the aged substratum in light-deprived tanks for 3 days. Moreover, although microalgae can grow heterotrophically, direct use of β -HB has not been demonstrated. Another probable reason for the decreasing level of total ammonia-nitrogen in PHB treatment could be the action of heterotrophic bacteria that convert ammonia-nitrogen directly to microbial biomass (Ebeling *et al.* 2006). Based on the abovementioned processes, further study is needed to investigate the component of the biofilm on the PHB substratum responsible for the decreasing levels of total ammonia-nitrogen. Further research on PHB substratum should also include quantification of the nitrogen balance.

6.6.3 PHB substratum as additional food source

Many studies have documented the utility of artificial substratum in enhancing fish production. Burford *et al.* (2004) reported that epiphytes contributed 39-53 % to shrimp requirements of carbon and nitrogen. Khatoon *et al.* (2007b) also observed that the specific growth of shrimp improved by 28 % in the presence of substratum, presumably due to the presence of additional food source. It was also demonstrated by Ballester *et al.* (2003) that the growth and survival of *F. paulensis* postlarvae were not enhanced in the presence of substratum with their biofilm periodically removed. This indicates the relevance of biofilm on the substratum as food source. In the present study, the increased survival either in postlarval giant tiger shrimp or whiteleg shrimp may be attributed either by PHB as food source by direct ingestion or the presence of biofilm on the surface.

6.7 Some limitations on the application of PHB as substratum in penaeid shrimp culture

Despite the current evidences on the beneficial effects of PHB biodegradable plastic as artificial substratum in penaeid culture, there have been several disadvantages observed in the present study: intrinsic brittleness that limits its processing particularly into rolls, highly biodegradable nature and high cost.

The intrinsic brittleness of PHB and its blends is recognized to be one of its limitations. Indeed, the elongation at break is very different between poly(3-hydroxybutyrate-co-3-hydroxyvalerat (PHBV) (< 15 %) and polypropylene (PP) (400 %) (El-Hadi *et al.* 2002). One reason for this is its low nucleation density and therefore large spherulites exhibit inter-spherulitic cracks (Bugnicourt *et al.* 2014). Strategies to improve the flexibility of PHB were described in Chapter 1 (section 1.6.5.1).

The highly biodegradable nature of PHB substratum observed in the present work provides an alternative source of food for either postlarval tiger shrimp or whiteleg shrimp by ingesting the degraded fractions, but at the same time, this nature presents major concern on long-term application in penaeid culture. Visual observation of the two types of PHB substrata (DP9002 and P209) used in the present work generally showed onset of biodegradation 5 days after introduction in raceways containing penaeid shrimp. The degrading PHB substratum is typically characterized having distended surface with microcracks appearing as shown in Figure 6.2.

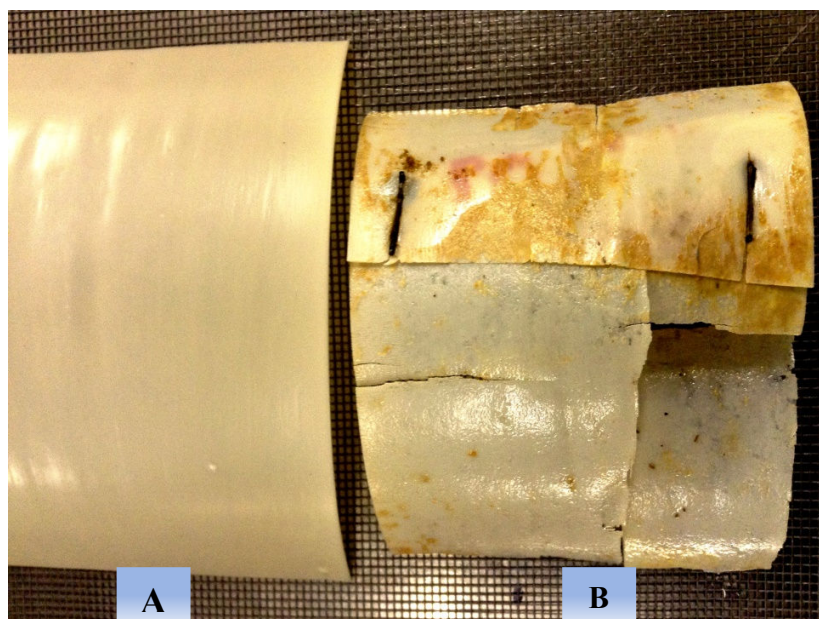


Figure 6.2 PHB-based bioplastic type P209: original material (A); material immersed in a recirculating tank of the Shrimp Facility of ARC for 15 days (B).

According to Spyros & Kimmich (1997), PHB and other PHAs contain amorphous and crystalline regions, of which the former are much more susceptible to microbial attack. The microbial degradation of the amorphous regions of PHB is characterized by rigidity and further degradation of the amorphous regions made the structure of the polymer much looser (Bonartsev *et al.* 2007) which were observed in the present work.

The high price of PHB is also seen as a major restriction for its application as artificial substratum. The current cost of PHB production is reported to be in the range of 2–5 € kg⁻¹ and this is considerably higher than major petrochemical polymers such as polyethylene (PE) and PP with production cost of approximately 1.2 € kg⁻¹ (Song *et al.*

2009). Castilho *et al.* (2009) have identified the cost of production materials, mainly the carbon sources, to account for as much as 50 % of the total operating cost, making it one of the most important determinants on the overall economics of PHB production. There have been several suggested strategies to lower the price of PHB production or PHAs in general: cheap carbon substrate for bacterial growth coming from by-products or waste materials, use of mixed culture or modified bacteria or microalga and optimization of extraction processes (Bugnicourt *et al.* 2014). An elegant strategy for production of a super PHA strain combined with advanced fermentation processes was proposed by Wang *et al.* (2014a). These authors also proposed a functional PHA strains with technology to control the precise structures of PHA molecules allowing for PHA with high value added applications. For PHB to be more useful in the future, its cost should in the order of conventional polymers.

6.8 General conclusions

The introduction of the bacterial storage compound poly- β -hydroxybutyrate (PHB) as new management strategy in penaeid shrimp has been explored in this research and its contribution is outlined in Figure 6.3.

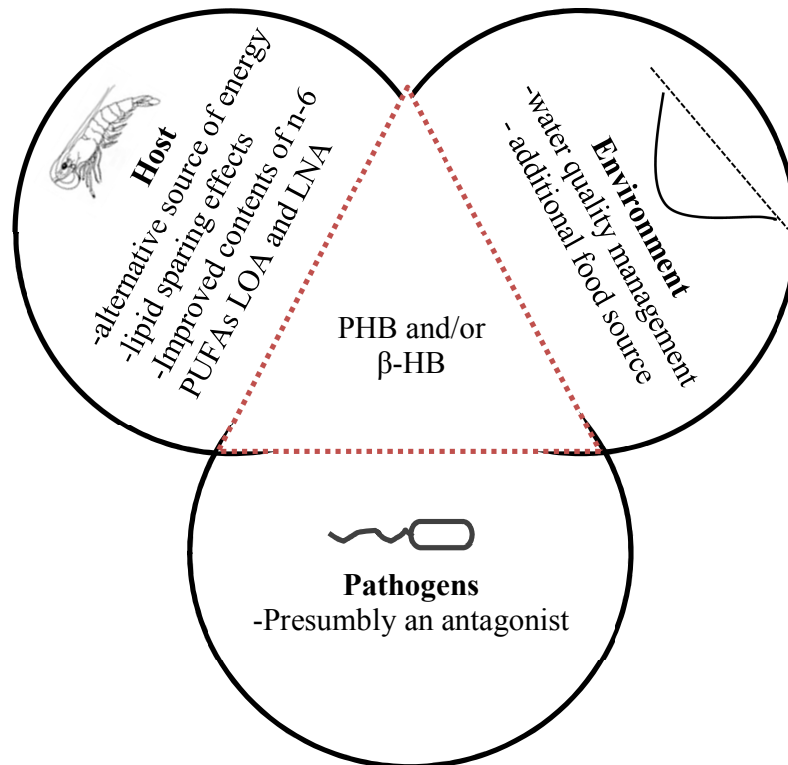


Figure 6.3. Contribution of PHB and/or β -HB in penaeid shrimp.

From the diagram, the application of PHB in penaeid shrimp has lead to the following:

- PHB either supplied via *Artemia* in early stages of tiger shrimp or offered as artificial substratum in postlarval stages of tiger shrimp and whiteleg shrimp enhanced its survival;
- PHB either supplied via *Artemia* in early stages of tiger shrimp or offered as artificial substratum in postlarval stages of tiger shrimp PHB could not be used to stimulate growth. Growth stimulation was observed for postlarval whiteleg shrimp when PHB was offered as artificial substratum;

- PHB supplied via *Artemia* enhanced the robustness of early stages of tiger shrimp against adverse environmental conditions;
- PHB supplied via *Artemia* enhanced the resistance of early stages of tiger shrimp against pathogenic bacteria;
- PHB and/or β -HB has a lipid-saving effects in starved *Artemia*;
- PHB and/or β -HB enhanced the contents of essential n-6 PUFAs LOA and LNA in starved *Artemia*;
- PHB and/or β -HB is probably used as energy source but not as building block;
- PHB offered as artificial substratum increased the survival and overall performance of penaeid shrimp;
- PHB offered as artificial substratum improved the water quality by decreasing the level of total ammonia-nitrogen.

Further research is however required to explore and understand the full potential of PHB as a biocontrol strategy in penaeid shrimp culture. This should focus on:

- degradation of PHB into β -HB in the gut of penaeid shrimp;
- degradation of PHB into β -HB in shrimp culture systems and its effects on the standing microbial community;
- mechanisms on the utilization of dietary PHB as alternative source of energy in the absence/presence of carbohydrates and/or protein as sources of energy in penaeid shrimp as shown in Figure 6.4.

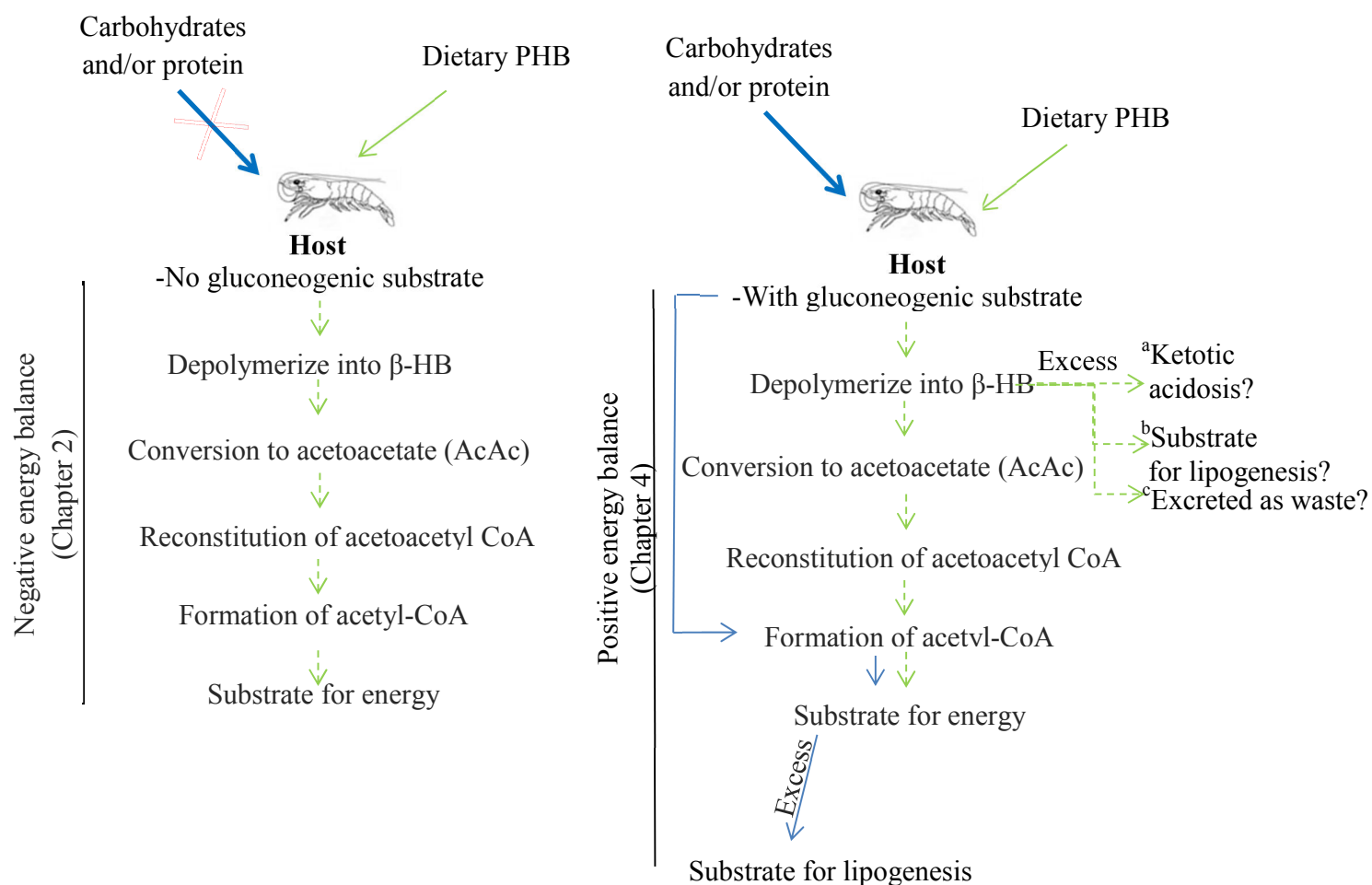


Figure 6.4 Proposed lines of studies pertaining to the utilization of dietary PHB in the absence/presence of carbohydrates and/or protein as primary sources of energy in penaeid shrimp. Broken arrow (\dashrightarrow) represents hypothetical conversion of PHB into β -HB monomers and its fate in penaeid shrimp. In the absence of carbohydrates and/or protein as primary sources of energy, β -HB, a ketone body, is used as alternative source of energy through ketolytic pathway as described by Laffel (1999). In the presence of carbohydrates and/or protein as primary sources of energy, dietary PHB and/or its depolymerized product

β -HB is still likely be used as substrate for energy, however, excessive production may result either in ketotic acidosis^(a) as reported by Weltzien *et al.* (2000), as substrate for lipogenesis^(b) or excreted in urine^(c). ^aIn *Artemia*, excessive levels of β -HB resulted in increased mortality, presumably due to a ketotic acidosis. This condition is associated with rapid and progressive rise in serum hydrogen ion concentration as β -HB is a strong organic acids that dissociate fully at physiological pH. This condition further outstrips the buffering capacity of the serum and tissues resulting in the development of ketotic acidosis (Laffel 1999).^b Although findings in Chapter 4 have indicated that PHB offered in natural matrix is not assimilated in the structural components (i.e. long-chain fatty acids) of postlarval *L. vannamei*, further study is needed to investigate this aspect. ^cIn vertebrates, excessive production of ketone bodies is excreted through the urine (Guyton 1991) and thus, it should be investigated whether aquatic farmed hosts have the capacity to excrete excessive β -HB a previously reported for vertebrates.

- supplementation of different doses of PHB to various developmental stages of penaeid shrimp including the quantification of the actual amount of PHB encapsulated in the livefeeds and as such passed on to the next level in the food chain;
- actual levels of β -HB in PHB-enriched livefeeds as well as in the larval predator and its potential adverse effects when present in excessive amounts;
- Energy and protein balance in PHB-enriched livefeeds as well as in the larval predator;

- *In vivo* tests to explore the direct effects of PHB against common pathogenic bacteria in penaeid shrimp;
- Assimilation of PHB in short and medium-chain length fatty acids in penaeid shrimp;
- mode of action of PHB on the gut-associated microbial biota in penaeid shrimp through application of molecular techniques such as DGGE;
- PHB and its monomer β -HB as immunostimulant and gene expression modulators in penaeid shrimp;
- component of the biofilm on the PHB substratum responsible for water quality management;
- effects of PHB substratum on the nitrogen balance in shrimp ponds;
- strategy to increase flexibility of PHB biodegradable plastic (e.g. comparison of different types of plastizers, determination of the appropriate levels and evaluation of its impacts on the environment);
- strategy to render PHB more effective, e.g. by integrating it into existing microbial control strategies as mentioned by De Schryver *et al.* (2010a).

REFERENCES

A

- Abe H. & Doi Y. (2002) Side-chain effect of second monomer units on crystalline morphology, thermal properties, and enzymatic degradability for random copolyesters of (R)-3-hydroxybutyric acid with (R)-3-hydroxyalkanoic acids. *Biomacromolecules* 3 (1): 133-138.
- Abraham T.J., Shanmugam S.A., Palaniappan R. & Dhevendaran K. (2003) Distribution and abundance of luminous bacteria with special reference to shrimp farming activities. *Indian Journal of Marine Sciences* 32 (3): 208-213.
- Akiyama D.M., Dominy W.G. & Lawrence A.L. (1991) Penaeid shrimp nutrition for the commercial feed industry: revised. *In: Proceedings of the Aquaculture Feed Processing and Nutrition Workshop, Thailand and Indonesia* (Akiyama D.M. & Tan R.K.H. eds.), pp. 80–98. American Soybean Association, Singapore.
- Akoh C.C. & Min D.B. (1998) Food lipids; chemistry, nutrition and biotechnology, Part IV. Marcel Dekker, New York, pp. 432–427.
- Alavi S., Thomas S., Sandeep K.P., Kalarikkal N., Varghese J. & Yaragalla S. (2014) *Polymers for packaging. Technology & Engineering*, CRC Press, 486 p.
- Alberts B., Johnson A., Lewis J., Raff M., Roberts K. & Walter P. (2002) *Molecular Biology of the Cell*. 4th edition. New York: Garland Science. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK26871/>.

- Alcaraz G., Espinoza V. & Vanegas C. (1999) Acute effect of ammonia and nitrite on respiration of *Penaeus setiferus* postlarvae under different levels. Journal of the World Aquaculture Society 30: 98–106.
- Alday-Sanz V., Roque A. & Turnbull J.F. (2002) Clearing mechanisms of *Vibrio vulnificus* biotype I in the black tiger shrimp *Penaeus monodon*. Diseases of Aquatic organisms 48: 91–99.
- Amache R., Sukan A., Safari M., Roy I. & Keshavarz T. (2013) Advances in PHAs Production. Chemical Engineering Transactions 32: 931-936.
- American Society for Testing and Materials (ASTM) (2000) Standard test methods for acidity or alkalinity of water: D 1067–92. In: Annual Book of ASTM Standards, Water and Environmental Technology. Section 11.01, pp. 65–71. American Society for Testing and Materials, West Conshocken, Pennsylvania, USA,
- Ansari S. & Fatma T. (2014) Polyhydroxybutyrate - A biodegradable plastic and its various formulations. 1st ed. New Delhi: International Journal of Innovative Research in Science, Engineering and Technology 3 (2): 9 598-9 602.
- Apines-Amar M.J.S. & Amar E.C. (2015) Use of immunostimulants in shrimp culture: An update. Biotechnological Advances in Shrimp Health Management in the Philippines, 45-71.
- Argue B.A., Arce S.M., Lotz J.M. & Moss S.M. (2002) Selective breeding of Pacific white shrimp (*Litopenaeus vannamei*) for growth and resistance to Taura Syndrome Virus. Aquaculture 204: 447-460.

- Arnold S.J., Sellars M.J., Crocos P.J. & Coman G.J. (2006) Intensive production of juvenile tiger shrimp *Penaeus monodon*: An evaluation of stocking density and artificial substrates. *Aquaculture* 261 (3): 890-896.
- Arnold S.J., Coman F.E., Jackson C.J. & Groves S. A. (2009) High-intensity, zero water-exchange production of juvenile tiger shrimp, *Penaeus monodon*: An evaluation of artificial substrates and stocking density. *Aquaculture* 293 (1–2): 42–48.
- Arts M. T. (1998) Ecophysiology of lipids in pelagic crustacean zooplankton communities. *In: Molecular approaches to the study of the ocean* (Cooksey K. E. ed.), pp. 329-341. Chapman and Hall, London.
- Audelo-Naranjo M., Martínez-Córdova L.R., Gómez-Jiménez S. & Voltolina D. (2012) Intensive culture of *Litopenaeus vannamei* without water exchange and with an artificial substrate. *Hidrobiológica* 22 (1): 1-7.
- Austin B. & Austin D.A. (1999) Bacterial fish pathogens, 3rd edition. Springer-Praxis, Chichester, UK, 457 p.
- Azim M.E., Wahab M.A., Verdegem M.C.J., Van Dam A.A., Van Rooij J.M. & Beveridge M.C.M. (2002) The effects of artificial substrates on freshwater pond productivity and water quality and the implications for periphyton-based aquaculture. *Aquatic Living Resources* 15 (4): 231–241.
- Azim M.E. & Wahab M.A. (2005) Periphyton-based pond polyculture. *In: Periphyton: Ecology, Exploitation and Management* (Azim M.E., Verdegem M.C.J., Van Dam A.A. & Beveridge M.C.M. eds.), pp. 207-222. CABI Publishing, Wallingford, UK.

B

- Bachere E. (2003) Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. *Aquaculture* 227: 427-438.
- Balakrishnan G., Peyail S., Ramachandran K., Theivasigamani A., Savji K.A., Chokkaiah M. & Nataraj P. (2011) Growth of cultured White leg shrimp *Litopenaeus Vannamei* (Boone 1931) in different stocking density. *Advances in Applied Science Research* 2: 107-113.
- Ballester E.L.C., Wasielesky W.J., Cavalli R.O., Santos M.H.S. & Abreu P.C. (2003) Influência do biofilme no crescimento do camarão-rosa *Farfantepenaeus paulensis* em sistemas de berçário. *Atlântica* 25: 117-122.
- Ballester E.L.C., Wasielesky W. Jr., Cavalli R.O. & Abreu P.C. (2007) Nursery of the pink shrimp *Farfantepenaeus paulensis* in cages with artificial substrates: Biofilm composition and shrimp performance. *Aquaculture* 269 (1–4): 355–362.
- Bamstedt U. (1986) Chemical composition and energy content. *In: The Biological Chemistry of Marine Copepods* (Corner E. & O'Hara S. ed.), pp. 1-58. Oxford University Press, Oxford.
- Barman D., Kumar V., Roy S. & Mandal S.C. (2012) Specific pathogen free shrimps: Their scope in aquaculture. *World Aquaculture Society* 43 (1): 67-68.
- Baruah K., Huy T.T., Norouzitallab P., Niu Y., Gupta S.K., De Schryver P. & Bossier P. (2015) Probing the protective mechanism of poly- β -hydroxybutyrate against

- vibriosis by using gnotobiotic *Artemia franciscana* and *Vibrio campbellii* as host-pathogen model. Scientific Reports 5: 9 427.
- Beeby M., Chob M., Stubbe J. & Jensen G.J. (2012) Growth and localization of polyhydroxybutyrate granules in *Ralstonia eutropha*. Journal of Bacteriology 194 (5): 1 092-1 099.
- Behiry S. I., Younes H. A., Mohamed M.H. & Mohamed A.A. (2015) Fatty acid polymorphism of *Ralstonia solanacearum* in different Egyptian governorates and other European countries. Plant Pathology Journal 14 (4): 202-206.
- Beis A., Zammit V.A. & Newsholme E.A. (1980) Activities of 3-hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase in relation to ketone-body utilisation in muscles from vertebrates and invertebrates. European Journal of Biochemistry 104 (1): 209-15.
- Bengtson D.A., Léger P. & Sorgeloos P. (1991) Use of *Artemia* as a food source for aquaculture. In: *Artemia Biology* (Browne R.A., Sorgeloos P. & Trotman C.N.A. eds), pp. 255–285. CRC Press Inc., Boca Raton, FL.
- Bermúdez-Almada M.C. & Espinosa-Plascencia A. (2012) The use of antibiotics in shrimp farming. In: *The use of antibiotics in shrimp farming, health and environment in aquaculture* (Carvalho E. ed.), pp. 199–214. InTech, www.intechopen.com.
- Bharti V., Pandey P.K., Purushothaman C.S. & Rajkumar M. (2013) Bacterial communities in biofilm and their effect on production of *Cirrhinus mrigala* (Hamilton, 1822). Aquaculture (Communicated).

- Biagi G., Piva A., Moschini M., Vezzali E. & Roth F.X. (2007) Performance, intestinal microflora, and wall morphology of weanling pigs fed sodium butyrate. *Journal of Animal Science* 85: 1 184-1 191.
- Biao X. & Kaijin Y. (2007) Shrimp farming in China: operating characteristics, environmental impact and perspectives. *Ocean and Coastal Management* 50: 538–550.
- Bligh E. G. & Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37 (8): 911–917.
- Bonartsev B.A.P., Myshkina V.L., Nikolaeva D.A., Furina E.K., Makhina T.A., Livshits V.A., Boskhomdzhiev A.P., Ivanov E.A., Iordanskii A.L. & Bonartseva G.A. (2007) Biosynthesis, biodegradation, and application of poly(3-hydroxybutyrate) and its copolymers: natural polyesters produced by diazotrophic bacteria. *In: Communicating Current Research and Educational Topics and Trends in Applied Microbiology* (Mendez-Vilas A. ed.), pp. 295–307, Formatex, Badajoz, Spain.
- Bondad-Reantaso M.G., Subasinghe R.P., Josupeit H., Cai J. & Zhou X. (2012) The role of crustacean fisheries and aquaculture in global food security: past, present and future. *Journal of Invertebrate Pathology* 110: 158–165.
- Boschker H.T.S. (2004) Linking microbial community structure and functioning: stable isotope (^{13}C) labeling in combination with PLFA analysis. *In: Molecular microbial ecology manual II*. (Kowalchuk G.A., De Bruijn F.J., Head I.M., Akkermans A.D. & Van Elsas J.D. eds), pp. 3 575-3 590. Kluwer Academic Publishers, Dodrecht, The Netherlands.

- Bratvold D. & Browdy C.L. (2001) Effects of sand sediment and vertical surfaces (AquaMatsTM) on production, water quality, and microbial ecology in an intensive *Litopenaeus vannamei* culture system. *Aquaculture* 195 (1-2): 81–94.
- Braunegg G., Lefebvre G. & Genser K.F. (1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. *Journal of Biotechnology* 65: 127-161.
- Bricknell I. & Dalmo R.A. (2005) The use of immunostimulants in fish larval aquaculture. *Fish Shellfish Immunology* 19: 457–472.
- British Nutrition Foundation (1992) Unsaturated fatty acids: Nutritional & physiological significance. The Report of the British Nutrition Foundation's Task Force, British Nutrition Foundation, London.
- Brock J.A. & Main K.L. (1994) A guide to the common problems and diseases of cultured *Penaeus vannamei*. World Aquaculture Society, Baton Rouge, Louisiana, USA, 242 p.
- Brock J.A., Gose R.B., Lightner D.V. & K.W. Hasson (1997) Recent developments and an overview of Taura syndrome of farmed shrimp in the Americas. *In: Diseases in Asian Aquaculture III* (Flegel T.W. & MacRae I.H. eds.), pp. 275–283. Proceedings from the Third Symposium on Diseases in Asian Aquaculture, Bangkok, Thailand.
- Brown E.E. (1983) *World Fish Farming: Cultivation and Economics*. 2nd ed., AVI Publishing, Westport, Connecticut.

- Brown M.R. (2002) Nutritional value of microalgae for aquaculture. *In: Avances en nutricion acuicola VI* (Cruz-Suarez L.E., Ricque-Marie D., Tapia-Salazar M., Gaxiola-Cortes M.G. & Simoes N. eds.) Memorias del VI Symposium Internacional de Nutricion Acuicola. 3-6th September, Cancun, Mexico.
- Bugnicourt E., Cinelli P., Lazzeri A. & Alvarez V. (2014) Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. *eXPRESS Polymer Letters* 8 (11): 791–808.
- Burford M.A., Sellars M.J., Arnold S.J., Keys S.J., Crocos P.J. & Preston N.P. (2004) Contribution of the natural biota associated with substrates to the nutritional requirements of the post-larval shrimp, *Penaeus esculentus* (Haswell), in high-density rearing systems. *Aquaculture Research* 35: 508–515.
- Burrell P.C., Phalen C.M. & Hovanec T.A. (2001) Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. *Applied and Environmental Microbiology* 67 (12): 5 791-5 800.
- Byrom D. (1994) Polyhydroxyalkanoates. *In: Plastics from microbes: microbial synthesis of polymers and polymer precursors* (Mobley D.P. ed.), pp. 5–33. Hanser, Munich.

C

- Cadogan D.F. & Howick C.J. (1996) Plasticizers. *Kirk-Othmer Encyclopedia of Chemical Technology*, John Wiley and Sons, New York, pp. 258–290.

- Camargo J.A., Alonso A. & Salamanca A. (2005) Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. *Chemosphere* 58 (9): 1 255–1 267.
- Castilho L.R., Mitchell D.A. & Freire D.M.G. (2009) Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology* 100: 5 996-6 009.
- Castille F., Lawrence A., Buisman P. & Drost R. (2004) Effects of sterol supplements (cholesterol FG, cholesterol SF, and sterols M1M) on growth and survival of the shrimp, *Litopenaeus vannamei* Boone. *In: Avances en Nutrición Acuícola VII. Memorias del VII Simposium Internacional de Nutrición Acuícola* (Cruz Suárez L.E., Ricque Marie D., Nieto López M.G., Villarreal D., Scholz U. & González M. eds.), pp. 504–517. Hermosillo, Sonora, México.
- Cavalli R.O., Vanden Berghe E., Lavens P., Thuy T.T.N., Wille, M. & Sorgeloos P. (2000) Ammonia toxicity as a criterion for the evaluation of larval quality in the prawn *Macrobrachium rosenbergii*. *Comparative Biochemistry and Physiology* 125: 333 – 343.
- Chamberlain P.M., Bull I.D., Black H.I.J., Ineson P. & Evershed R.P. (2004) Lipid content and carbon assimilation in Collembola: implications for the use of compound-specific carbon isotope analysis in animal dietary studies. *Oecologia* 139: 325–335.
- Chamberlain G.W. (2010) History of shrimp farming. *In: The Shrimp Book*, 1st ed. (Alday-Sanz V. ed.), pp. 1-34. Nottingham University Press, Nottingham, U.K.

- Chandge M. S. & Paulraj R. (1998) Requirements of linoleic and linolenic acid in the diet of Indian white prawn *Penaeus indicus* (H Milne Edwards). Indian Journal of Marine Sciences 27: 402-406.
- Chang C.F., Chen H.Y., Su M.S. & Liao I.C. (2000) Immunomodulation by dietary beta-1, 3-glucan in the brooders of the black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunology 10: 505-514.
- Chang H.M., Wang Z.H., Luo H.N., Xu M., Ren X.Y., Zheng G.X., Wu B.J., Zhang X.H., Lu X.Y., Chen F., Jing X.H., & Wang L. (2014) Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)-based scaffolds for tissue engineering. Brazilian Journal Of Medical and Biological Research 47 (7): 533–539.
- Chanratchakool P. & Phillips M.J. (2002) Social and economic impacts and management of shrimp disease among small-scale farmers in Thailand and Vietnam. *In: Primary Aquatic Animal Health Care in Rural, Small-scale, Aquaculture Development* (Arthur J.R., Phillips M.J., Subasinghe R.P., Reantaso M.B. & MacRae I.H. eds.), FAO Fisheries Technical Paper No. 406.
- Chen J.C. & Lei S.C. (1990) Toxicity of ammonia and nitrite to *Penaeus monodon* juveniles. Journal of World Aquaculture Society 21 (4): 300–306.
- Chen G.Q. & Wu Q. (2005) The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26 (33): 6 565-6 578.
- Chen G.Q. (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chemical Society Reviews 38: 2 434–2 446.

- Cheng W. & Chen J. (1998) *Enterococcus*-like infections in *Macrobrachium rosenbergii* are exacerbated by high pH and temperature but reduced by low salinity. *Diseases of Aquatic Organisms* 34: 103-108.
- Cherrington C.A., Hinton M., Mead G. & Chopra I. (1991) Organic acids: Chemistry, antibacterial activity and practical applications. *Advances in Microbial Physiology* 32: 87–108.
- Chien Y.W. (1992) Water quality requirements and management for marine shrimp culture. *In: Proceedings of the Special Session on Shrimp Farming* (Wyban J.M. ed.), pp. 145–156. The World Aquaculture Society, Baton Rouge, Louisiana.
- Choi G.G., Kim H.W. & Rhee Y.H. (2004) Enzymatic and non-enzymatic degradation of poly (3-Hydroxybutyrate-co-3-hydroxyvalerate) copolyesters produced by *Alcaligenes* sp. MT-16. *Journal of Microbiology* 42: 346-352.
- Chung H., Lee O., Huang Y., Mok S., Kolter R. & Qian P. (2010) Bacterial community succession and chemical profiles of subtidal biofilms in relation to larval settlement of the polychaete *Hydroides elegans*. *International Society for Microbial Ecology* 4 (6): 817-828.
- Cock J., Gitterle T., Salazar M. & Rye M. (2009) Breeding for disease resistance of Penaeid shrimps. *Aquaculture* 286: 1–11.
- Colt J.E. & Armstrong D.A. (1981) Nitrogen toxicity to crustaceans, fish and molluscs. *In Proceedings of the Bio-Engineering Symposium for Fish Culture* (Allen L.J. & Kinney E.C. eds.), pp. 34-67. Fish Culture Section of the American Fisheries Society, Bethesda, Maryland.

- Costa R., Mermoud I., Kobravi S., Morlet B., Haffner P., Berthe F., Legroumellec M. & Grimont P. (1998) Isolation and characterization of bacteria associated with a *Penaeus stylirostris* disease (Syndrome 93) in New Caledonia. *Aquaculture* 164: 297- 309.
- Couch J.A. (1978) Diseases, parasites, and toxic responses of commercial penaeid shrimps of the Gulf of Mexico and South Atlantic coasts of North America. *Fishery Bulletin* 76: 1-44.
- Coutteau P. & Mourente G. (1997) Lipid classes and their content of n – 3 highly unsaturated fatty acids (HUFA) in *Artemia franciscana* after hatching, HUFA-enrichment and subsequent starvation. *Marine Biology* 130: 81–91.
- Curé K., Gajardo G. & Cotteau P. (1996) The effects of DHA/EPA ratio in livefeed on the fatty acid composition, survival, growth and pigmentation of turbot larvae *Scophthalmus maximus* L. – Improvement of the Commercial Production of Marine Aquaculture Species: Proceedings of a workshop on Fish and Mollusc Larviculture, pp. 108–118.
- Cuzon G., Rosas C., Gaxiola G., Taboada G. & Van Wormhoudt A. (2000) Utilization of carbohydrates by shrimp. *In: Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola* (Cruz-Suárez L.E., Ricque-Marie D., Tapia-Salazar M., Olvera-Novoa M.A., Civera-Cerecedo R. eds.), pp. 328–339. Mérida, Yucatán.

D

- D'Abramo L.R. (1989) Lipid requirements of shrimp. *Advances in Tropical Aquaculture*. AQUACOPIFREMER, Tahiti, pp. 271–285.
- Daniels C.L., Merrifield D.L., Boothroyd D.P., Davies S.J., Factor J.R. & Arnold K.E. (2010) Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture* 304: 49–57.
- Davis D.A. (2005) Nutrient Requirements of Penaeid Shrimp. *Global Aquaculture Advocate* 8 (4): 72-74.
- Dedkova E.N. & Blatter L.A. (2014) Role of β -hydroxybutyrate, its polymer poly- β -hydroxybutyrate and inorganic polyphosphate in mammalian health and disease. *Frontiers in Physiology* 5 (260): 1-22.
- Defoirdt T., Halet D., Sorgeloos P., Bossier P. & Verstraete W. (2006) Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. *Aquaculture* 261: 804-808.
- Defoirdt T., Boon N., Sorgeloos P., Verstraete W., Bossier P. (2007a) Alternatives to antibiotics to control bacterial infections-luminescent vibriosis in aquaculture as an example. *Trends in Biotechnology* 25 (10): 472–479.
- Defoirdt T., Halet D., Vervaeren H., Boon N., Van de Wiele T., Sorgeloos P., Bossier P. & Verstraete W. (2007b) The bacterial storage compound poly-beta-hydroxybutyrate

- protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. Environmental Microbiology 9 (2): 445-52.
- Defoirdt T., Boon N., Sorgeloos P., Verstraete W., & Bossier P. (2009) Short-chain fatty acids and poly- β -hydroxyalkanoates: (New) Biocontrol agents for a sustainable animal production. Biotechnology Advances 6: 680-685.
- Defoirdt T., Sorgeloos P. & Bossier P. (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology 14: 251–258.
- De Koning G.J.M & Lemstra P.J. (1992) The amorphous state of bacterial poly[(R)-3-hydroxyalkanoate] in vivo. Polymer 33: 3 292–3 294.
- Denef K., Roobroeck D., Wadu M.C.W.M., Lootens P. & Boeckx P. (2009) Microbial community composition and rhizodeposit-carbon assimilation in differently managed temperate grassland soils. Soil Biology and Biochemistry 41 (1): 144–153.
- Department of Commerce, Bureau of the Census. (2015) Shrimp Q Yearly. <http://www.ers.usda.gov/data-products/aquaculture-data.aspx>.
- De Schryver P. (2010a) Poly-beta-hydroxybutyrate as a microbial agent in aquaculture. PhD thesis, Ghent University, Belgium.
- De Schryver P., Sinha A., Kunwar P., Baruah K., Verstraete W., Boon N., De Boeck G. & Bossier P. (2010b) Poly-beta-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Applied Microbiology and Biotechnology 86: 1 535-1 541.

- De Schryver P., Defoirdt T. & Sorgeloos P. (2014) Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? PLoS Pathogens 10 (4): e1003919. doi:10.1371/journal.ppat.1003919.
- De Troch M., Boeckx P., Cnudde C., Van Gansbeke D., Vanreusel A. Vincx M. & Caramujo M.J. (2012) Bioconversion of fatty acids at the basis of marine food webs: insights from a compound-specific stable isotope analysis. Marine Ecology Progress Series 465: 53–67.
- Dhont J., Lavens P. & Sorgeloos P. (1991) Development of alipid-enrichment technique for *Artemia* juveniles in an intensive system for use in marine larviculture. *In*: Larvi '91. Short communications and abstracts of contributions presented at the International Symposium on Fish and Crustacean Larviculture (Lavens P. *et al.* eds.), pp. 51–55. European Aquaculture Society Special Publication, Gent, Belgium.
- Dinakaran G.K., Soundarapandian P. & Varadharajan D. (2013) Tail Rot Disease in *Macrobrachium idella idella* (Hilgendorf, 1898). Journal of Drug Metabolism & Toxicology 4: 155.
- Drakeford B. & Pascoe S. (2008) Substitutability of fishmeal and fish oil in diets for salmon and trout: A meta-analysis. Aquaculture Economics & Management 12: 155–175.

E

- Ebeling J.M., Timmons M.B. & Bisogni J.J. (2006) Engineering analysis of the stoichiometry of photoautotrophic, autotrophic and heterotrophic removal of ammonia-nitrogen in aquaculture systems. *Aquaculture* 257 (1-4): 346-358.
- Ebnesajjad S. (2012) Plastic films in food packaging: Materials, technology and applications. Elsevier, 384 p.
- El-Hadi A., Schnabel R., Straube E., Müller G. & Henning S. (2002) Correlation between degree of crystallinity, morphology, glass temperature, mechanical properties and biodegradation of poly(3-hydroxyalkanoate) PHAs and their blends. *Journal of Polymer Testing* 21 (6): 665-674.
- Erlandson J.M. (2001) The archaeology of aquatic adaptations: Paradigms for a new millennium. *Journal of Archaeological Research* 9: 287–350.

F

- Faimali M., Garaventa F., Terlizzi A., Chiantore M. & Cattaneo-Vietti R. (2004) The interplay of substrate nature and biofilm formation in regulating *Balanus amphitrite* Darwin, 1854 larval settlement. *Journal of Experimental Marine Biology and Ecology* 306 (1): 37-50.
- FAO (2000) The State of World Fisheries and Aquaculture 2000. FAO, Rome, Italy.
- FAO (2001) Tropical shrimp fisheries and their impact on living resources. FAO Fisheries

Circular 974, FIIT/C974. Rome.

FAO (2002*a*) World review of fisheries and aquaculture. Rome, Italy.

FAO (2002*b*) Farming freshwater prawn: A manual for the culture of the giant tiger prawn (*Macrobrachium rosenbergii*). FAO Fisheries Technical Paper, Rome Italy, 219 p.

FAO (2005) Introduction and movement of two penaeid shrimp species in Asia and the Pacific. FAO Fisheries Technical Paper 476 (also available at www.fao.org/docrep/009/a0086e/A0086E00.htm).

FAO (2009) The State of the World Fisheries and Aquaculture 2008. Rome.
<http://www.fao.org/docrep/011/i0250e/i0250e00.htm>.

FAO (2013*a*) FishStatJ, a tool for fishery statistics analysis.
<http://www.fao.org/fishery/statistics/software/fishstatj/en>.

FAO (2013*b*) Report of the FAO/MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPND) of Cultured Shrimp (under TCP/VIE/3304). FAO Fisheries and Aquaculture Report No. 1053. Rome. 54 p.

FAO (2014*a*) The State of World Fisheries and Aquaculture. Rome, Italy.

FAO (2014*b*) Aquaculture Feed and Fertilizer Resources Information System (AFFRIS). FAO, Rome, Italy (available at: <http://www.fao.org/fishery/affris/species-profiles/en>).

FAO (2015) Wild and Aquaculture Capture Production 1950–2013. FishStat J software and databases. Available online at <http://www.fao.org/fishery/statistics/software/fishstat/en>.

- Fast A.W. & Menasveta P. (2000) Some recent issues and innovations in marine shrimp pond culture. *Reviews in Fisheries Science* 8 (3): 151-233.
- Feigon L. (2000) A harbinger of the problems confronting China's economy and environment: the great Chinese shrimp disaster of 1993. *Journal of Contemporary China* 9: 323–332.
- Fenucci J.L., Lawrence A.L. & Zein-Eldin Z.P. (1981) The effects of fatty acid and shrimp meal composition of prepared diets on growth of juvenile shrimp, *Penaeus stylirostris*. *Journal of the World Mariculture Society* 12: 315-324.
- Flegel T.W. (1997) Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World Journal of Microbiology and Biotechnology* 13: 433–442.
- Flegel T.W. & Pasharawipas T. (1998) Active viral accommodation: A new concept for crustacean response to viral pathogens. *In: Advances in Shrimp Biotechnology* (Flegel T. W. ed.), pp. 245-250. National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.
- Flegel T.W. (2006) Disease testing and treatment. *In: Operating procedures for shrimp farming. Global Shrimp OP Survey Results and Recommendations* (Boyd C.E., Jory D. & Chamberlain G.W. eds.), pp. 98–103. Global Aquaculture Alliance, St. Louis, Missouri, USA.
- Flegel T.W., Lightner D.V., Lo C.F. & Owens L. (2008) Shrimp disease control: past, present and future. *In: Diseases in Asian Aquaculture VI* (Bondad-Reantaso M.G., Mohan C.V., Crumlish M., Subasinghe R.P. eds.), pp. 355–378. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Folch J., Lees M. & Sloane-Stanley G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* 226(1): 497–509.

G

GAA (Global Aquaculture Alliance) (2001) Codes of Practice for Responsible Shrimp Farming. St. Louis, Missouri, USA.

GAA (Global Aquaculture Alliance) (2003) SPF, SPR, SPT-Status Understanding the Health Condition Of Aquaculture Organisms. Retrieved from: <http://pdf.gaalliance.org/pdf/GAA-Alday-Feb03.pdf>.

GAA (Global Aquaculture Alliance) (2013) Cause of EMS shrimp disease identified. GAA News Releases. Available: <http://www.gaalliance.org/newsroom>

Galeotti M. (1998) Some aspects of the application of immunostimulants and a critical review of methods for their evaluation. *Journal of Applied Ichthyology* 14: 189-199.

Garcia B., Olivera E.R., Minambres B., Fernandez-Valverde M., Canedo L.M., Prieto M.A., Garcia J.L., Martinez M., Luengo J.M. (1999) Novel biodegradable aromatic plastics from a bacterial source. Genetic and biochemical studies on a route of the phenylacetyl-CoA catabolon. *Journal of Biological Chemistry* 274 (41): 29 228–29 241.

- Gebauer B. & Jendrossek D. (2006) Assay of poly(3-Hydroxybutyrate) depolymerase activity and product determination. *Applied and Environmental Microbiology* 72 (9): 6 094–6 100.
- Gibson G.R. & Roberfroid M.B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition* 125: 1 401–1 412.
- Glencross B.D. & Smith D.M. (1999) The dietary linoleic and linolenic fatty acids requirements of the prawn, *Penaeus monodon*. *Aquaculture Nutrition* 5: 53–63.
- Glencross B.D., Smith D.M., Thomas M.R. & Williams K.C. (2002). The effect of dietary n–3 and n–6 fatty acid balance on the growth of the prawn *Penaeus monodon*. *Aquaculture Nutrition* 8 (1): 43–52.
- Gogolewski S., Jovanovic M., Perren S.M., Dillon J.G. & Hughes M.K. (1993) Tissue response and *in vivo* degradation of selected polyhydroxyacids: Polylactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/VA). *Journal of Biomedical Materials Research* 27 (9): 1 135–1 148.
- Gomez-Gil B., Tron-Mayen L., Roque A., Turnbull J.F., Inglis V. & Guerra-Flores A.L. (1996) Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. *Aquaculture* 163:1-9.
- Gomez-Gil B., Roque A. & Turnbull J.F. (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* 191: 259–270.

- Gong H., Lawrence A.L., Gatlin D.M., Jiang D.H. & Zhang F. (2001) Comparison of different types and levels of commercial soybean lecithin supplemented in semipurified diets for juvenile *Litopenaeus vannamei* Boone. *Aquaculture Nutrition* 7: 11–17.
- González-Félix M.L. & Perez-Velazquez M. (2002) Current status of lipid nutrition of Pacific white shrimp *Litopenaeus vannamei*. In: *Advances on Nutricion Acuicola VI. Memorias del VI Simposium International de Nutricion Acuicola* (Cruz-Surez L.E., Ricque-Marie D., Tapia-Salazar M., Gaxiola-Cortés M.G. & Simoes N. eds.), pp. 35–45. Cancun, Quintana Roo, Mexico.
- Gräslund S., Bergqvist P.A. & Bengtsson B.E. (1999) Potential formation of toxic by-products from the use of chlorine as a disinfectant in intensive shrimp farming in South East Asia (abstract). 4th Symposium on Disease in Asian Aquaculture, Cebu City, Philippines.
- Gräslund S. & Bengtsson B.E. (2001) Chemicals and biological products used in South-east Asian shrimp farming, and their potential impact on the environment—a review. *Science of the Total Environment* 280 (1–3): 93–131.
- Graszynski K. (1970) Ketonkörperstoffwechsel beim Flußkrebs: Stoffwechselwege, organverteilung und physiologische bedeutung. *Zeitschrift für Vergleichende Physiologie* 66 (4): 439–462.
- Guary J.C., Kayama M., Murakami Y. & Ceccaldi H.J. (1976) The effects of a fat-free diet and compounded diets supplemented with various oils on molt, growth and fatty acid composition of prawn, *Penaeus japonicus* Bate. *Aquaculture* 7 (3): 245–254.

Guillaume J. (2001) Nutrition and feeding of fish and crustaceans. Springer–Praxis Books in Aquaculture and Fisheries. London, Springer.

Guyton A.C. (1991) Textbook of Medical Physiology, 8th ed. W.B. Saunders, London, p. 1014.

H

Hai N.V. & Fotadar R. (2009) Comparison of the effects of the prebiotics (Bio-Mos® and [beta]-1,3-D-glucan) and the customised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). Aquaculture 289 (3-4): 310-316.

Halet D., Defoirdt T., Van Damme P., Vervaeren H., Forrez I., Van de Wiele T., Boon N., Sorgeloos P., Bossier P. & Verstraete W. (2007) Poly-beta-hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. FEMS Microbiology Ecology 60: 363-369

Han K., Geurden I. & Sorgeloos P. (2000) Enrichment strategies for *Artemia* using emulsions providing different levels of n-3 highly unsaturated fatty acids. Aquaculture 183: 335–34.

Handrick R., Reinhardt S., Kimmig P., & Jendrossek D. (2004) The “intracellular” poly(3-hydroxybutyrate) (PHB) depolymerase of *Rhodospirillum rubrum* is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases. Journal of Bacteriology 186 (21): 7 243–7 253.

- Hauge K.H., Cleeland B. & Wilson D.C. (2009) Fisheries Depletion and Collapse. Rep. International Relief Governance Council, Web. 4 Mar. 2013. http://irgc.org/wpcontent/uploads/2012/04/Fisheries_Depletion_full_case_study_web.pdf.
- Heinzelmann S.M., Bale N. J., Hopmans E.C., Damsté J.S.S., Schouten S. & Van der Meer M.T.J. (2014) Critical Assessment of glyco- and phospholipid separation by using silica chromatography. *Applied and Environmental Microbiology* 80 (1): 360-365.
- Hernández-Rodríguez A., Alceste-Oliviero C., Sanchez R., Jory D., Vidal L. & Constain-Franco L.F. (2001) Aquaculture development trends in Latin America and the Caribbean. *In: Aquaculture in the Third Millennium* (Subasinghe R.P., Bueno P., Phillips M.J., Hough C., McGladdery S.E. & Arthur J.R. eds.), pp. 317-340. Technical Proceedings of the Conference on Aquaculture in the Third Millennium, Bangkok, Thailand.
- Hertrampf J.W. (1991) Feeding aquatic animals with phospholipids. 1. Crustaceans. Lucas Meyer, Publication 8: 31 p.
- Holmström K., Gräslund S., Wahlström A., Pongshompoo S., Bengtsson B.E. & Kautsky N. (2003) Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science and Technology* 38: 255-266.
- Hoseinifar S.H., Zare P. & Kolangi Miandare H. (2015) The effects of different routes of inulin administration on gut microbiota and survival rate of Indian white shrimp post-larvae (*Fenneropenaeus indicus*). *Veterinary Research Forum* 6 (4): 331-335.

- Hung O.S., Thiyagarajan V. & Qian P.Y. (2008) Preferential attachment of barnacle larvae to natural multi-species biofilms: does surface wettability matter? *Journal of Experimental Marine Biology and Ecology* 361 (1): 36-41.
- Hung L.T. & Quy O.M. (2013) On farm feeding and feed management in whiteleg shrimp (*Litopenaeus vannamei*) farming in Viet Nam. *In: On-farming feeding and feed management in aquaculture* (Hasan M.R. & New M.B. eds.), pp. 337–357. FAO Fisheries and aquaculture technical paper No. 583. Rome, Italy.
- Hung N.V., De Schryver P., Tam T.T., Garcia-Gonzalez L., Bossier P. & Nevejan N. (2015) Application of poly- β -hydroxybutyrate (PHB) in mussel larviculture. *Aquaculture* 446: 318–324.

I

- Iba W., Rice M.A. & Wikfors G.H. (2014) Microalgae in Eastern Pacific White Shrimp, *Litopenaeus vannamei* (Boone 1931) hatcheries: A review on roles and culture environments. *Asian Fisheries Science* 27: 212-233.

J

- Jayasree L., Janakiram P. & Madhavi R. (2006) Characterization of *Vibrio* spp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). *Journal of World Aquaculture Society* 37: 523-532.

- Jendrossek D., Frisse A., Behrends A., Andermann M., Kratzin H.D. & Stanislawski T. (1995) Biochemical and molecular characterization of the *Pseudomonas lemoignei* polyhydroxyalkanoate depolymerase system. *Journal of Bacteriology* 177: 596–607.
- Jendrossek D. & Handrick R. (2002) Microbial degradation of polyhydroxyalkanoates *Annual Review of Microbiology* 56: 403-432.
- Jendrossek D., Selchow O. & Hoppert M. (2007) Poly(3-Hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in *Caryophanon latum*. *Applied and Environmental Microbiology* 73 (2): 586-593.
- Jie K., Qingwen Z., Ping L. & Jianhai X. (2003) Selective breeding raises WSSV resistance in fleshy prawns. *Global Aquaculture Advocate*, pp. 25–26.
- Johnson K., Kleerebezem R. & Van Loosdrecht M.C.M. (2010) Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. *Water Research* 44: 2 141–2 152.

K

- Kaiser G.E. & Wheaton F.W. (1983) Nitrification filters for aquatic culture systems: state of the art. *Journal of World Mariculture Society* 14: 302–324.
- Kalia V.C., Lal S. & Cheema S. (2007) Insight in to the phylogeny of polyhydroxyalkanoate biosynthesis: horizontal gene transfer. *Gene* 389 (1): 19-26.

- Kanazawa A., Teshima S., Ono K. & Chalayondeja K. (1979). Biosynthesis of fatty acids from acetate in the prawn, *Penaeus monodon* and *P. merguensis*. Memoirs of the Faculty of Fisheries, Kagoshima University 28: 21–26.
- Kaplan D.L. (2013) Biopolymers from renewable resources. Springer Science & Business Media, Berlin Heidelberg, New York, 420 p.
- Karunasagar I., Shivu M.M., Girisha S.K., Krohne G. & Karunasagar I. (2007) Biocontrol of pathogens in shrimp hatcheries using bacteriophages. Aquaculture 268 (1–4): 288–292.
- Kashket E.R. (1987) Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiology Reviews 46 (3): 233–244.
- Kasuya K., Doi Y. & Yao T. (1994) Enzymatic degradation of poly[(R)-3-hydroxybutyrate] by *Comamonas testosteroni* ATSU of soil bacterium. Polymer Degradation and Stability 45: 379–386.
- Kautsky N., Ronnback P., Tedengren M. & Troell M. (2000) Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture 191: 145-161.
- Kayama M., Hirata M., Kanazawa A., Tokiwa S. & Saito M. (1980) Essential fatty acids in the diet of prawn-III. Lipid metabolism and fatty acid composition. Bulletin of the Japanese Society for the Science of Fish 46: 483-488.
- Khanna S. & Srivastava A.K. (2005) Recent advances in microbial polyhydroxyalkanoates. Process Biochemistry 40: 607–619.

- Khatoon H., Yusoff F., Banerjee S., Shariff M. & Bujang J.S. (2007a) Formation of periphyton biofilm and subsequent biofouling on different substrates in nutrient enriched brackishwater shrimp ponds. *Aquaculture* 273 (4): 470–477.
- Khatoon H., Yusoff F. M., Banerjee S., Shariff M. & Mohamed S. (2007b) Use of periphytic cyanobacterium and mixed diatoms coated substrate for improving water quality, survival and growth of *Penaeus monodon* Fabricius postlarvae. *Aquaculture* 271: 196–205.
- Kimura M., Kobayashi K., Matsuoka A., Hayashi K. & Kimura Y. (1985) Head-space gas-chromatographic determination of 3-hydroxybutyrate in plasma after enzymic reactions, and the relationship among the three ketone bodies. *Clinical Chemistry* 31 (4): 596-598.
- Kimura T., Yamano K., Nakano H., Momoyama K., Hiraoka M. & Inouye K. (1996) Detection of penaeid rod-shaped DNAvirus (PRDV) by PCR. *Fish Pathology* 31 (2): 93–98.
- Kiran G.S., Lipton A.N., Priyadharshini S., Anitha K., Cruz-Suárez L. E., Arasu M.V., Choi K.C., Selvin J. & Al-Dhabi N. A. (2014) Antiadhesive activity of poly-hydroxy butyrate biopolymer from a marine *Brevibacterium casei* MSI04 against shrimp pathogenic vibrios. *Microbial Cell Factories* 13: 114.
- Klein A.H., Wendroth S.M., Drewes L.R. & Andrews M.T. (2010) Small-volume d-β-hydroxybutyrate solution infusion increases survivability of lethal hemorrhagic shock in rats. *Shock* 34 (6): 565-72.

- Kobayashi M. & Melkonyan T. (2011) Strategic incentives in biosecurity investment: Theoretical and empirical analyses. *Journal of Agricultural and Resource Economics* 36 (2): 242-262.
- Kolakowska A., Olley J. & Dunstan G.A. (2002) Fish lipids. *In: Chemical and functional properties of food lipids* (Sikorski Z.E. & Kolakowska A. eds.), pp. 221–264. CRC Press, FL, USA.
- Kopinke F.D., Remmler M. & Mackenzie K. (1996) Thermal decomposition of biodegradable polyesters - I: Poly(b-hydroxybutyric acid). *Polymer Degradation and Stability* 52 (1): 25-38.
- Korsatko W., Wabnegg B., Tillian H.M., Braunegg G. & Lafferty R.M. (1983) Poly-D-(-)-3-hydroxybutyric acid: a biodegradable carrier for long term medication dosage. II. Comm.: the biodegradation in animal organism and in-vitro - in-vivo correlation of the liberation of pharmaceuticals from parenteral matrix retard tablets. *Pharmazeutische Industrie* 45 (10): 1 004–1 007.
- Koyama N. & Doi Y. (1995) Morphology and biodegradability of a binary blend of poly((R)-3-hydroxybutyric acid) and poly((R,S)-lactic acid). *Canadian Journal of Microbiology* 41 (1): 316-322.
- Kungvankij P., Tiro L.B.J., Pudadera B.J.J., Potestas I.O., Corre K.G., Borlongan E., Talean G.A., Bustilo L.F., Tech E.T., Unggui A. & Chua T. E. (1986) Shrimp hatchery design, operation and management. Bangkok, Thailand: Network of Aquaculture Centres in Asia.

- Kureshy N. & Davis D.A. (2000) Metabolic requirements for protein by pacific white shrimp, *Litopenaeus vannamei*. In: Avances en Nutricion Acuicola V. Memorias del V Simposium de Nutricion Acuicola (Cruz-Suarez L.E., Ricque-Marie D., Tapia-Salazar M.A., Civera-Cerecedo R. eds.), pp.161–180. Merida, Yucatan.
- Kusaka S., Abe H., Lee S.Y. & Doi Y. (1997) Molecular mass of poly[(R)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. Applied Microbiology and Biotechnology 47: 140–143.

L

- Laffel L. (1999) Ketone Bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes/Metabolism Research and Reviews 15 (6): 412-426.
- Lafferty K.D., Harvell C.D., Conrad J.M., Friedman C.S., Kent M.L., Kuris A.M., Powell E.N., Rondeau D., & Saksida S.M. (2015) Infectious diseases affect marine fisheries and aquaculture economics. Annual Review of Marine Science 7: 471-496.
- Lananan F., Jusoh A., Ali N.A., Lam S.S. & Endut A. (2013) Effect of conway medium and f/2 medium on the growth of six genera of South China Sea Marine Microalgae. Bioresource Technology 141: 75–82.
- Langis R., Proulex D., De la Noue J. & Couture P. (1988) Effects of bacterial biofilm on intensive *Daphnia* culture. Aquaculture Engineering 7: 21-38.

- Laranja J.L., Ludevese-Pascual G.L. Amar E.C., Sorgeloos P., Bossier P. & De Schryver P. (2014) Poly- β -hydroxybutyrate (PHB) accumulating *Bacillus* spp. improve the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae. *Veterinary Microbiology* 173 (3-4): 310-317.
- Larkin T. (2012) Aquaculture global outlook and sustainability. *In*: 24th Annual North Carolina Aquaculture Development Conference, Raleigh, North Carolina, USA.
- Lavilla-Pitogo C.R. (1995) Bacterial diseases of penaeid shrimps: an Asian view. *In*: Diseases in Asian Aquaculture II (Shariff M., Arthur J.R. & Subasinghe R.P. eds.), pp.107-121. Fish Health Section, Asian Fisheries Society, Manila, Philippines.
- Leaf A., Kang J.X. & Xiao Y.F. (2008) Fish oil fatty acids as cardiovascular drugs. *Current Vascular Pharmacology* 6: 1–12.
- LeBlanc P.J. & Ballantyne (2000) Novel aspects of the activities and subcellular distribution of enzymes of ketone body metabolism, in the liver and kidney of the goldfish, *Carassius auratus*. *Journal of Experimental Zoology* 286: 434–439.
- Lee M. H. & Shiau S. Y. (2002) Dietary vitamin C and its derivatives affect immune responses in grass shrimp, *Penaeus monodon*. *Fish Shellfish Immunology* 12 (2): 119-129.
- Lee M. H. & Shiau S. Y. (2004) Vitamin E requirements of juvenile grass shrimp, *Penaeus monodon*, and effects on non-specific immune responses. *Fish Shellfish Immunology* 16 (4): 475-485.

- Léger P. & Sorgeloos P. (1992) Optimized feeding regimes in shrimp hatchery. *In: Marine Shrimp Culture: Principle and Practices* (Fast E.W. & Lester L.J. eds), pp. 225–244. Elsevier Sciences Publisher BV, Amsterdam, The Netherlands.
- Le Moullac G. & Haffner P. (2000) Environmental factors affecting immune responses in Crustacea. *Aquaculture* 191: 121–131.
- Lenz R.W. & Marchessault R.H. (2005) Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. *Biomacromolecules* 6: 1–8.
- Lepage G. & Roy C.C. (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *Journal of Lipid Research*. 25 (12): 1 391–1 396.
- Li P., Wang X., Murthy S., Gatlin III D.M., Castille F.L., & Lawrence A.L. (2009). Effect of dietary supplementation of brewer's yeast and Grobionic- A on growth, immune responses, and low-salinity tolerance of pacific white shrimp *Litopenaeus vannamei* cultured in recirculating systems. *Journal of Applied Aquaculture* 21: 110e119.
- Liao I.C. & Liu F.G. (1989) A brief review of nutritional studies for *Penaeus monodon*. *In: Advances in tropical aquaculture, Actes de Colloque*, 9, pp. 355–380. AQUACOP IFREMER, Plouzané, France.
- Liao I.C. (2000) The use of chemicals in aquaculture in Taiwan, Province of China. *In: Proceedings of the meeting on the use of chemicals in aquaculture in Asia*

- (Arthur J.R., Lavilla-Pitogo C.R. & Subasinghe R.P. eds.), pp. 193–207. Southeast Asian Fisheries Development Center, Tigbauan, Philippines.
- Lightner D.V. (1983) Diseases of cultured penaeid shrimp. *In*: Crustacean Aquaculture Volume 1 (McVey J.P. ed.), pp. 289–320. CRC Handbook of Mariculture. CRC Press, Boca Raton, FL, USA.
- Lightner D.V. (1996) Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. *Revue scientifique et technique* (International Office of Epizootics) 15: 579–601.
- Lightner D.V., Redman R.M., Arce S.M. & Moss S.M. (2009) Specific pathogen-free shrimp stocks in shrimp farming facilities as a novel method for disease control in crustacean. *In*: Shellfish Safety and Quality (Shumway S. & Rodrick G. eds.), pp. 384–424. Woodhead Publishing Limited, Cambridge, England.
- Lightner D. V. (2011) Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review. *Journal of Invertebrate Pathology* 106(1): 110–130.
- Ling S.W. (1977) Aquaculture in Southeast Asia: a historical review. University Washington Press, Seattle, Washington. 108 p.
- Lin Y.C., Chen J.C., Morni W.Z.W., Putra D.F., Huang C.-L., Li C.-C. & Hsieh J.F. (2013) Vaccination enhances early immune responses in white shrimp *Litopenaeus vannamei* after secondary exposure to *Vibrio alginolyticus*. *PLoS ONE* 8 (7): 1-15.
- Lithner D. (2011) Environmental and health hazards of chemicals in plastic polymers and products. PhD Thesis. Department of Plant and Environmental Sciences, University of Gothenburg, Sweden.

- Liu Y., De Schryver P., Van Delsen B., Maignien L., Boon N., Sorgeloos P., Verstraete W., Bossier P., Defoirdt T. (2010) PHB-degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent vibriosis. *FEMS Microbiology Ecology* 74: 196–204.
- Loc-Carrillo C. & Abedon S.T. (2011) Pros and cons of phage therapy. *Bacteriophage* 1: 111–114.
- Lotz J.M. (1997) Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. *World Journal of Microbiology and Biotechnology* 13 (4): 405-413.

M

- Ma D., Hu Y., Wang J., Ye S. & Li A. (2006) Effects of antibacterials use in aquaculture on biogeochemical processes in marine sediment. *The Science of the Total Environment* 367 (1): 273-277.
- Madison L.L. & Huisman G.W. (1999) Metabolic engineering of poly(3 hydroxyalkanoates): from DNA to plastic. *Microbiology and Molecular Biology Review* 63 (1): 21-53.
- Mahious A.S. & Ollevier F. (2005) Probiotics and prebiotics in aquaculture. 1st regional Workshop on Techniques for Enrichment of Live Food for use in Larviculture. AAARC, Urmia, Iran, pp. 67.
- Mahious A.S., Gatesoupe F.J., Hervi M., Metailler R. & Ollevier F. (2006) Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus C. 1758). *Aquaculture International* 14 (3): 219- 229.

- Makridis P. & Vadstein O. (1999) Food size selectivity of *Artemia franciscana* at three developmental stages. *Journal of Plankton Research* 21: 2 191-2 201.
- Mark H.F. (2013) *Encyclopedia of Polymer Science and Technology, Concise*. John Wiley & Sons, 1350 p.
- Martinez-Porchas M. & Martinez-Cordova L.R. (2012) World aquaculture: environmental impacts and troubleshooting alternatives. *Scientific World Journal* Article ID 389623. <http://dx.doi.org/10.1100/2012/389623>.
- Martins T.G., Cavalli R.O., Martino R.C., Rezende C.E.M & Wasielesky W. (2006) Larviculture output and stress tolerance of *Farfantepenaeus paulensis* postlarvae fed *Artemia* containing different fatty acids. *Aquaculture* 252 (2-4): 525-533.
- Marques A. , Dhont J., Sorgeloos P. & Bossier P. (2004) Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp *Artemia franciscana*. *Journal of Experimental Marine Biology and Ecology* 312 (1): 115–136.
- Marques A., Dinh T., Ioakeimidis C., Huys G., Swings J., Verstraete W., Dhont J., Sorgeloos P. & Bossier P. (2005) Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. *Applied and Environmental Microbiology* 71 (80): 4 307–4 317.
- Matavulj M. & Molitoris H.P. (1992) Fungal degradation of polyhydroxyalkanoates and a semiquantitative assay for screening their degradation by terrestrial fungi. *FEMS Microbiology Reviews* 9 (2-4): 323–331.

- McVey J.P. (1993) CRC Handbook of mariculture, Volume I. Crustacean Aquaculture, Second Edn. CRC Press.
- Merican Z.O. & Shim K.F. (1996) Qualitative requirements of essential fatty acids for juvenile *Penaeus monodon*. Aquaculture 147 (3-4): 275–291.
- Meurant G. (1990) Advances in Marine Biology Volume 27. Academic Press, 504 p.
- Merrick J. M., Lundgren D. G. & Pfister R.M. (1965) Morphological changes in poly- β -hydroxybutyrate granules associated with decreased susceptibility to enzymatic hydrolysis. Journal of Bacteriology 89: 234-239.
- Middelburg J.J., Barranguet C., Boschker H.T.S., Herman P.M.J., Moens T. & Heip C.H.R. (2000) The fate of intertidal microphytobenthos carbon: an in situ C13-labeling study. Limnology and Oceanography 45 (6): 1 224–1 234.
- Millamena O.M. & Bangcaya J.P. (2001) Reproductive performance and larval quality of pond-raised *Scylla serrata* females fed various broodstock diets. Asian Fisheries Science 14: 153–159.
- Moss S.M. (1999) Biosecure shrimp production: emerging technologies for a maturing industry. Global Aquaculture Advocate 2: 50–52.
- Motoh H. & Buri P. (1981) Identification of postlarvae of the Genus *Penaeus* appearing in shore waters. Researches on Crustacea 11: 86-94.
- Mourente G., Medina A., Gonzalez S. & Rodriguez A. (1995) Variations in lipid content and nutritional status during larval development of the marine shrimp *Penaeus kerathurus*. Aquaculture 130 (2-3): 187-199.

- Mozumder M.S.I., De Wever H., Volcke E.I.P. & Garcia-Gonzalez L. (2014) A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production. *Process Biochemistry* 155: 272–280.
- Mukai K., Doi Y., Sema Y. & Tomita K. (1993) Substrate specificities in hydrolysis of polyhydroxyalkanoates by microbial esterases. *Biotechnology Letters* 15 (6): 601–604.
- Mulier K. E., Lexcen D.R., Luzcek E., Greenberg J.J. & Beilman Gregory J. (2012) Treatment with beta-hydroxybutyrate and melatonin is associated with improved survival in a porcine model of hemorrhagic shock. *Resuscitation* 83 (2): 253–258.
- Musthaq S.S. & Kwang J. (2011) Oral vaccination of baculovirus-expressed VP28 displays enhanced protection against white spot syndrome virus in *Penaeus monodon*. *PLoS One* 6: 1-9.

N

- Naik P. (2010) *Essentials of Biochemistry (for medical students)*. JP Medical Ltd, New Delhi India, 464 p.
- Najdegerami E.H., Tran T.N., Defoirdt T., Marzorati M., Sorgeloos P., Boon N. & Bossier P. (2012) Effects of poly-beta-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community. *FEMS Microbiology Ecology* 79 (1): 25–33.
- Najdegerami E.H., Baruah K., Shiri A., Rekecki A., Van den Broeck W., Sorgeloos P., Boon N., Bossier P. & De Schryver P. (2015) Siberian sturgeon (*Acipenser baerii*)

- larvae fed *Artemia* nauplii enriched with poly- β -hydroxybutyrate (PHB): effect on growth performance, body composition, digestive enzymes, gut microbial community, gut histology and stress tests. *Aquaculture Research* 46 (4): 801–812.
- Namikoshi A., Wu J. L., Yamashita T., Nishizawa T., Nishioka T., Arimoto M. & Muroga K. (2004) Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. *Aquaculture* 229: 25-35.
- Nemutanzhela M. E., Roets Y., Gardiner N. & Lalloo R. (2014). The use and benefits of *Bacillus* based biological agents in aquaculture. *In: Sustainable aquaculture techniques* (Hernandez-Vergar M. ed.), pp. 1-33. Murcia Spain.
- Newman S.G. (2009) Specific pathogen-free status advances shrimp culture. *Global Aquaculture Advocate*, 79-80.
- Nhan D.T., Wille M., De Schryver P., Defoirdt T., Bossier P. & Sorgeloos P. (2010) The effect of poly- β -hydroxybutyrate on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture* 302 (1-2): 76–81.
- Niquette P., Servais P. & Savoir R. (2000) Impacts of pipe materials on densities of fixed bacterial biomass in a drinking water distribution system. *Water Research* 34 (6): 1 952-1 956.
- Niu Y., Defoirdt T., Rekecki A., De Schryver P., Van de Broeck W., Dong S., Sorgeloos P., Boon N. & Bossier P. (2012) A method for the specific detection of resident bacteria in brine shrimp larvae. *Journal of Microbiological Methods* 89 (1): 33–37.

O

- Otoshi C.A., Montgomery A.D., Matsuda E. M. & Moss S. M. (2006) Effects of artificial substrate and water source on growth of juvenile Pacific White Shrimp, *Litopenaeus vannamei*. Journal of World Aquaculture 37 (2): 210-213.
- Owen O.E., Morgan A.P., Kemp H.G., Sullivan J.M., Herrera M.G. & Cahill G.F. Jr. (1967) Brain metabolism during fasting. Journal of Clinical Investigation 46 (10): 1 589–1 595.

P

- Pachekoski W.M., Agnelli J.A. M. & Belem L.P. (2009) Thermal, mechanical and morphological properties of poly (hydroxybutyrate) and polypropylene blends after processing. Materials Research Bulletin 12 (2): 159-164.
- Pandey P.K., Bharti V. & Kumar K. (2014) Biofilm in aquaculture production. African Journal of Microbiology Research 8 (13): 1 434-1 443.
- Parado-Estepa F.D., Qunitio E.T. & Borlongan E. (1991) Prawn hatchery operations. Aquaculture Manual no. 19. SEAFDEC/AQD, Tigbauan, Iloilo. 44 p.
- Parrish C.C. (2009) Essential fatty acids in aquatic food webs. *In*: Lipids in Aquatic (Arts M.T., Brett M.T. & Kainz M. eds.), pp. 309–326. Springer, New York.
- Parrish C.C. (2013) Lipids in marine ecosystems. ISRN Oceanography, 1–16.

- Patterson E., Wall R., Fitzgerald G.F, Ross R.P. & Stanton C. (2012) Health implications of high dietary omega-6 polyunsaturated fatty acids. *Journal of Nutrition and Metabolism* 2012: 539426. doi: 10.1155/2012/539426.
- Perez-Velazquez M., González-Félix M.L. & Lawrence A.L. (2004) Advances in triglyceride, fatty acid nutrition in penaeid shrimp. *Global Aquaculture Advocate*, 76–77.
- Pokrant B. (2014) Brackish water shrimp farming and the growth of aquatic monocultures in coastal Bangladesh. *Historical Perspectives of Fisheries Exploitation in the Indo-Pacific* 12: 107–132.
- Porter M.M. & Yu J. (2011) Crystallization kinetics of poly(3-Hydroxybutyrate) granules in different environmental conditions. *Journal of Biomaterials and Nanobiotechnology* 2: 301-310.
- Pradeep B., Pandey P.K. & Ayyappan S. (2003) Effect of probiotics and antibiotics on the water quality and bacterial flora. *Journal of Inland Fisheries Society of India* 35 (2): 68-72.
- Primavera J.H. (1994) Shrimp farming in the Asia-Pacific: Environmental and trade issues and regional cooperation. East Asia Science and Security Network (EASSNet). <http://nautilus.org/eassnet/shrimp-farming-in-the-asia-pacific-environmental-and-trade-issues-and-regional-cooperation/>
- Primavera J.H. (1997) Socioeconomic impacts of shrimp culture. *Aquaculture Research* 28 (10): 815–827.

Pruder G.D. (2004) Biosecurity: Application in aquaculture. *Aquacultural Engineering* 32: 3-10.

Puskas J.E. (2013) Introduction to polymer chemistry: A biobased approach. Technology & Engineering, DEStech Publications, Inc. 338 p.

Q

Qi Z., Zhang X.H., Boon N. & Bossier P. (2009) Probiotics in aquaculture of China — current state, problems and prospect. *Aquaculture* 290: 15–21.

Qiu Y.Z., Han J., Guo J.J. & Chen G.Q. (2005) Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from gluconate and glucose by recombinant *Aeromonas hydrophila* and *Pseudomonas putida*. *Biotechnology Letters* 27: 1 381-1 386.

R

Racotta I.S. & Hernández-Herrera R. (2000) Metabolic responses of the white shrimp, *Penaeus vannamei*, to ambient ammonia. *Comparative Biochemistry and Physiology* 125: 437–443.

Ramesh M.R., Shankar K.M., Mohan C.V. & Varghese T.J. (1999) Comparison of three plant substrates for enhancing carp growth through bacterial biofilm. *Aquaculture Engineering* 19: 119-131.

- Raviv S., Parnes S. & Sagi A. (2008) Coordination of reproduction and molt in decapods. *In: Reproductive biology of crustaceans* (Mente E. ed.), pp. 365-390. Enfield, NH: Science Publishers.
- Ravuru D.B. & Mude J. N. (2014) Growth of cultured white of shrimp *Litopenaeus vannamei* (Boone 1931) of brackish water culture system in summer season with artificial diet. *Advances in Applied Science Research* 5 (2): 25-28.
- Rebufat F. (2007) The old man and the sea: a necessary change of course. *Research EU*, pp. 5-6.
- Recirculating Farms Coalition (2013) From out of the blue, green farming. http://www.recirculatingfarms.org/wpcontent/uploads/2013/06/RFCreport_FINAL-FINAL.pdf.
- Reddy C.S.K, Ghai R., Rashmi & Kalia V.C.(2003) Polyhydroxyalkanoates: an overview. *Bioresource Technology* 87 (2): 137–146.
- Reed L.J. & Muench H. (1938) A simple method of estimating fifty percent endpoints. *The American Journal of Hygiene* 27: 493–497.
- Rengpipat S., Phianphak W., Piyatiratitivorakul S. & Menasveta P. (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* 167: 301–313.
- Renner L.D. & Weibel D. B. (2011) Physicochemical regulation of biofilm formation. *MRS Bulletin* 36 (5): 347-355.
- Reusch R.N. (1992) Biological Complexes of poly- β -hydroxybutyrate. *FEMS Microbiology Reviews* 103: 119–130.

- Ricke S.C. (2003) Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Science* 82: 632-639.
- Ritar A.J., Dunstan G.A., Crear B.J. & Brown M.R. (2003) Biochemical composition during growth and starvation of early larval stages of cultured spiny lobster (*Jasus edwardsii*) phyllosoma. *Comparative Biochemistry and Physiology* 136A: 353–370.
- Rivera-Pérez C., García-Carreño F.L. & Saborowski R. (2010) Purification and biochemical characterization of digestive lipase in Whiteleg shrimp. *Marine Biotechnology* 13 (2): 284–295.
- Rönnbäck P. (2001) Shrimp Aquaculture — State of the Art. Swedish EIA Centre Report, 91-576-6113-8, 1, Swedish University of Agricultural Sciences, Uppsala.
- Rønnestad I., Helland S. & Lie Ø. (1998) Feeding *Artemia* to larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.) results in lower larval vitamin A content compared with feeding copepods. *Aquaculture* 165 (1-2): 159-164.
- Rosas C., Cuzon G., Goxiola G., Arena L., Lemaire P., Soye C. & Van Wormhoudt A.V. (2000) Influence of dietary carbohydrate on the metabolism of juvenile *Litopenaeus stylirostris*. *Journal of Experimental Marine Biology and Ecology* 249: 181–198.
- Roque A., Molina-Aja A., Bolán-Mejía C. & Gomez-Gil B. (2001) In vitro susceptibility to 15 antibiotics of vibrios isolated from penaeid shrimps in Northwestern Mexico. *Journal of Antimicrobial Agents* 17: 383–387.
- Rosenberry A. L. (1999) *World Shrimp Farming*. Island Press 202 p.

- Rostkowski K.H., Criddle C.S. & Lepech M.D. (2012) Cradle-to-gate life cycle assessment for a cradle-to-cradle cycle: Biogas-to-bioplastic (and back). *Environmental Science and Technology* 46 (18): 9 822–9 829.
- Ruangpan L., Danayadol Y., Direkbusarakom S., Sriurairatana S. & Flegel T.W. (1999) Lethal toxicity of *Vibrio harveyi* to cultivated *Penaeus monodon* induced by a bacteriophage. *Diseases of Aquatic Organisms* 35: 195–201.
- Rudnik E. (2010) *Compostable Polymer Materials*. Elsevier, 224 p.
- Rütters H., Sass H., Cypionka H. & Rullkotter J. (2002) Phospholipid analysis as a tool to study complex microbial communities in marine sediments. *Journal of Microbiological Methods* 48 (2-3): 149–160.

S

- Sakai M. (1999) Current research status of fish immunostimulants. *Aquaculture* 172: 63-92.
- Sakai M. (2015) Fish immunostilumants. *Journal of Fisheries Sciences.com* 9 (4): 39-40.
- Sánchez-Paz A., García-Carreño F., Muhlia-Almazán A., Peregrino-Uriarte A.B., Hernández-López J. & Yepiz-Plascencia G. (2006) Usage of energy reserves in crustaceans during starvation: Status and future directions. *Insect Biochemistry and Molecular Biology* 36 (4): 241–249.
- Sanders J.K.M. (1993) NMR of nature's plastics and spider's webs: chemistry, physics or biology? *Chemical Society Reviews* 22: 1–7.

- Sandifer P.A. & Joseph J.D. (1976) Growth responses and fatty acid composition of juvenile prawns (*Macrobrachium rosenbergii*) fed a prepared ration augmented with shrimp head oil. *Aquaculture* 8: 128-138.
- Sandifer P.A., Hopkins J. S. & Stokes A. D. (1987) Intensive culture potential of *Penaeus vannamei*. *Journal of the World Aquaculture Society* 18 (2): 94–100.
- Saulnier D., Haffner P., Goarant C., Levy P. & Ansquer D. (2000) Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture* 191: 133–144.
- Schade D.S. & Eaton R.P. (1979) The regulation of plasma ketone body concentration by counterregulatory hormones in man, III: effects of norepinephrine in man. *Diabetes* 28: 5–10.
- Segner H., Dolle A. & Bohm R. (1997) Ketone body metabolism in the carp *Cyprinus carpio*: biochemical and ¹H NMR spectroscopical analysis. *Comparative Biochemistry and Physiology Part B* 116 (2): 257–262.
- Shah D., Londhe V. & Parikh R. (2011) Can levamisole alone maintain the immunity? *International Journal of Pharmacy and Pharmaceutical Sciences* 3 (2): 161-164.
- Shamsuzzaman M.M. & Biswas T.K. (2012) Aqua chemicals in shrimp farm: a study from south-west coast of Bangladesh. *Egyptian Journal of Aquatic Research* 38 (4): 275–285.
- Shiau S.Y. & Peng C.Y. (1992) Utilization of different carbohydrates at different dietary protein levels in grass prawn, *Penaeus monodon*, reared in seawater. *Aquaculture* 101: 241–250.
- Shiau S.Y. (1998) Nutrient requirements of penaeid shrimp. *Aquaculture* 164: 77–93.

- Shieh M.S. (1969) The biosynthesis of phospholipids in the lobster, *Homarus americanus*. Comparative Biochemistry and Physiology 30: 179-184.
- Shinomiya M., Iwata T., Kasuya K.-I. & Doi Y. (1997) Cloning of the gene for poly(3-hydroxybutyric acid) depolymerase of *Comamonas testosteroni* and functional analysis of its substrate-binding domain. FEMS Microbiology Letters 154: 89-94.
- Shiva V. & Karir G. (1997) Towards sustainable aquaculture. Research Foundation for Science, New Dehli, India. pp. 133.
- Shivakumar S. (2013) Poly-beta-hydroxybutyrate (PHB) depolymerase from *Fusarium solani* Thom. Journal of Chemistry, Article ID 406386.
- Shivaram C.M. & Raj R.P. (1997) Dietary lipid requirements of the juveniles of Indian white prawn *Penaeus indicus* H. Milne Edwards. Journal of Aquaculture in the Tropics 12(3): 165–180.
- Situmorang M.L. (2015) Application of poly-β-hydroxybutyrate in growth and health promotion of Nile tilapia *Oreochromis niloticus* culture. PhD thesis, Ghent University, Belgium.
- Situmorang M.L., De Schryver P., Dierckens K., & Bossier P. (2016) Effect of poly-β-hydroxybutyrate on growth and disease resistance of Nile tilapia *Oreochromis niloticus* juveniles. Veterinary Microbiology 182 (15): 44–49.
- Sivakumar K. & Felix S. (2011) Immunostimulants in the immune response of *Penaeus monodon* (Fabricius). Journal of the Marine Biological Association in India 53 (1): 58–67.

- Smith D. M., Tabrett S. J. & Barclay M.C. (2001) Cholesterol requirement of subadult black tiger shrimp *Penaeus monodon* (Fabricius). *Aquaculture Research* 32: 399–405.
- Smith V.J., Brown J.H. & Hauton C. (2003) Immunostimulation in crustaceans: does it really protect against infection? *Fish & Shellfish Immunology* 15: 71–90.
- Smith R. (2005) Biodegradable polymers for industrial applications. CRC Press, 532 p.
- Solis N.B. (1988) Biology and Ecology. *In: Biology and culture of Penaeus monodon*. Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan Iloilo Philippines.
- Soltanian S. (2007) Protection of gnotobiotic *Artemia* against *Vibrio campbelli* using baker's yeaststrains and extracts. PhD thesis. Ghent University. Belgium.
- Song J. H., Murphy R.J., Narayan R. & Davies G.B.H. (2009) Biodegradable and compostable alternatives to conventional plastics. *Philosophical transactions of the Royal Society of London.B: Biological Sciences* 364 (1 526): 2 127–2 139.
- Soonthornchai W., Rungrasamee W., Karoonuthaisiri N., Jarayabhand P., Klinbunga S., Söderhäll K. & Jiravanichpaisal P. (2010) Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Developmental and Comparative Immunology* 34: 19–28.
- Soto-Rodríguez S., Armenta M. & Gomez-Gil B. (2006) Effects of enrofloxacin and florfenicol on survival and bacterial population in an experimental infection with luminescent *Vibrio campbellii* in shrimp larvae of *Litopenaeus vannamei*. *Aquaculture* 255: 48-54.

- Speers-Roesch B., Robinson J.W. & Ballantyne J.S. (2006) Metabolic organization of the spotted ratfish, *Hydrolagus coliei* (Holocephali: Chimaeriformes): Insight into the evolution of energy metabolism in the chondrichthyan fishes. *Journal of Experimental Zoology* 305A: 631–644.
- Spyros A. & Kimmich R. (1997) ¹H NMR imaging study of enzymatic degradation in poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Evidence for preferential degradation of the amorphous phase by PHB depolymerase B from *Pseudomonas lemoignei*. *Macromolecules* 30: 8 218-8 225.
- Stickney R.R. (2000) History of Aquaculture. *In: Encyclopedia of Aquaculture* (Stickney R.R. ed.), pp. 436–446. John Wiley & Sons, New York, NY.
- Strom M., Paranjpye R.N. & Nilsson W.B. (2013) Pathogen update: *Vibrio* species. *Advances in Microbial Food Safety* 1: 97–113.
- Sudesh K., Abe H. & Doi Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science* 25 (10): 1 503–1 555.
- Suguna P., Binuramesh C., Abirami P., Saranya V., Poornima K., Rajeswari V. & Shenbagarathai R. (2014) Immunostimulation by poly- β hydroxybutyrate-hydroxyvalerate (PHB-HV) from *Bacillus thuringiensis* in *Oreochromis mossambicus*. *Fish and Shellfish Immunology* 36 (1): 90-97.
- Sui L., Cai J., Sun H., Wille M. & Bossier P. (2012) Effect of poly- β -hydroxybutyrate on Chinese mitten crab, *Eriocheir sinensis*, larvae challenged with pathogenic *Vibrio anguillarum*. *Journal of Fish Diseases* 35 (5): 359-364.

- Sui L., Liu Y., Sun H., Wille M., Bossier P. & De Schryver P. (2014) The effect of poly-b-hydroxybutyrate on the performance of Chinese mitten crab (*Eriocheir sinensis* Milne-Edwards) zoea larvae. *Aquaculture Research* 45: 558–565.
- Summavielle T., Monteiro P.R.R., Reis-Henriques M.A. & Coimbra J. (2003) In vitro metabolism of steroid hormones by ovary and hepatopancreas of the crustacean Penaeid shrimp *Marsupenaeus japonicus*. *Scientia Marina* 67 (3): 299-306.

T

- Takeuchi T. & Murakami K. (2007) Crustacean nutrition and larval feed, with emphasis on Japanese spinylobster, *Panulirus japonicus*. *Bulletin of Fisheries Research Agency* 20: 15-23.
- Tan G.Y.A., Chen C.L., Li L., Ge L., Wang L., Razaad I. M.N., Li Y., Zhao L., Mo Y. & Wang J.Y. (2014) Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers* 6 (3): 706–754.
- Tansey J.T., Sztalryd C., Gruia-Gray J., Roush D.L., Zee J.V., Gavrilova O., Reitman M.L., Deng C.X., Li C., Kimmel A.R. & Londos C. (2001) Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proceedings of the National Academy of Sciences, USA* 98: 6 494–6 499.
- Teshima S. (1997) Phospholipids and sterols. *In: Crustacean Nutrition* (D'Abramo L., Akiyama D.M. & Conklin D.E. eds.), pp. 85-107. World Aquaculture Society, Baton Rouge, LA, USA.

- Thai T.Q., Wille M., Garcia-Gonzalez L., Sorgeloos P., Bossier P. & De Schryver P. (2014) Poly- β -hydroxybutyrate content and dose of the bacterial carrier for *Artemia* enrichment determine the performance of giant freshwater prawn larvae. *Applied Microbiology and Biotechnology* 98 (11): 5 205-5 215.
- Thompson J.M. & Bergersen E.P. (1991) Role of size, conditioning and lipid content in the overwinter survival of age-0 Colorado squawfish. *Transactions of the American Fisheries Society* 120: 346–353.
- Thompson F.L., Abreu P.C. & Cavalli R. (1999) The use of microorganisms as food source for *Penaeus paulensis* larvae. *Aquaculture* 174 (1–2): 139–153.
- Thompson F.L., Abreu P.C. & Wasielesky W. (2002) Importance of biofilm for water quality and nourishment in intensive shrimp culture. *Aquaculture* 203 (3–4): 263–278.
- Thompson R.C., Moore C.J., vom Saal F.S. & Swan S.H. (2009) Plastics, the environment and human health: current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences* 364: 2 153–2 166.
- Tidwell J. H., Coyle D.C. & Schulmeiste G. (1998) Effects of added substrate on the production and population characteristics of freshwater prawns *Macrobrachium rosenbergii* in ponds. *Journal of the World Aquaculture Society* 29 (1): 17-22.
- Tidwell J.H., Coyle S., Weibel C. & Evans J. (1999) Effects and interactions of stocking density and added substrate on production and population structure of freshwater prawn *Macrobrachium rosenbergii*. *Journal of the World Aquaculture Society* 30 (2): 174-179.

- Tidwell J.H. & Allan G.L. (2001) Fish as food: aquaculture's contribution: Ecological and economic impacts and contributions of fish farming and capture fisheries. European Molecular Biology Organization (EMBO) Reports 2 (11): 958-963.
- Tidwell J. & Coyle S. (2008) Impact of substrate physical characteristics on grow-out of freshwater prawn, *Macrobrachium rosenbergii*, in ponds and pond microcosm tank. Journal of the World Aquaculture Society 39 (3): 406-413.
- Tocher D.R. & Glencross B.D. (2015) Lipids and fatty acids. *In*: Dietary Nutrients, Additives and Fish Health (Cheng-Sheng L. ed.), pp. 47-94. John Wiley and Sons, Hoboken, New Jersey, USA.
- Tokiwa Y. & Ugwu C.U. (2007) Biotechnological production of (R)-3-hydroxybutyric acid monomer. Journal of Biotechnology 132: 264-272.
- Tran L., Nunan L., Redman R. M., Mohny L. L., Pantoja C. R., Fitzsimmons K. & Lightner D. V. (2013) Determination of the infectious nature of the agent of Acute Hepatopancreatic Necrosis Syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms 105: 45–55.
- Treece G.D. & Fox J.M. (1999) Design, operation and training manual for an intensive culture shrimp hatchery. Fish hatcheries, DIANE Publishing, 187 p.
- Treerattrakool S., Panyim S., Chan S.M., Withyachumnarnkul B. & Udomkit A. (2008) Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. Journal for the Federation of European Biochemical Societies 275: 970-980.

Tsokos J., Kreisberg R., Michaels A., Komm B. & Linton J. (1983) Respiratory and calcium transport properties of spiny lobster hepatopancreas mitochondria. Archives of Biochemistry and Biophysics 224 (2): 707-17.

U

United Nations. World Population Prospects: The 2010 Revision, Vol. 1, Comprehensive Tables. New York: United Nations; 2011. <http://esa.un.org/unpd/wpp/index.htm>.

V

Valipour A., Shariatmadari F., Abedian A., Seyfabadi S. J. & Zahmatkesh A.A (2011) Growth, molting and survival response of juvenile narrow clawed crayfish, *Astacus leptodactylus*, fed two sources of dietary oils. Iranian Journal of Fisheries Sciences 10 (3): 505-518.

Van Dam A.A., Beveridge M.C.M., Azim M.E. & Verdegem M.C.J. (2002) The potential of fish production based on periphyton. Reviews in Fish Biology and Fisheries 12 (1): 1–31.

Van Stappen G. (1996) Introduction, biology and ecology of *Artemia*. In: Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper (Lavens P. & Sorgeloos P. eds.), pp. 79–106. FAO, Rome, Italy.

Varadharajan D. & Pushparajan N. (2013) Food and feeding habits of aquaculture candidate a potential crustacean of Pacific White shrimp *Litopenaeus vannamei*,

- South East Coast of India. Journal of Aquaculture Research and Development 4:161.
- Vaseeharan B., Jayakumar R. & Ramasamy P. (2003) PCR based detection of white spot syndrome virus in cultured and captured crustaceans in India. Letters in Applied Microbiology 37: 443-447.
- Vasile C. & Zaikov G.E. (2009) Environmentally degradable materials based on multicomponent polymeric systems. Koninklijke Brill NV, Leiden.
- Vasylchenko O.A., Yastremskaya L.S., Salata A.M., Salata O.S. & Fedun N.O. (2011) Biotechnological aspects of poly- β -hydroxybutyrate biosynthesis. Biotechnologia Acta 3: 9-17.
- Venegas C.A., Nonaka L., Mushiake K., Nishizawa T. & Muroga K. (2000) Quasi-immune response of *Penaeus japonicus* to penaeid rod-shaped DNA virus (PRDV). Diseases of Aquatic Organisms 42: 83-89.
- Verschuere L., Rombaut G., Huys G., Dhont J., Sorgeloos P. & Verstraete W (1999) Microbial control of the culture of *Artemia* juveniles through pre-emptive colonization by selected bacterial strains. Applied and Environmental Microbiology 65 (6): 2 527-2 533.
- Verschuere L., Rombaut G., Sorgeloos P. & Verstraete W. (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64: 655–671.

- Vijayan K.K., Stalin R.V., Balasubramanian C.P., Alavandi S.V., Thillai S.V & Santiago T.C. (2005) Polychaete worms- a vector for white spot syndrome virus (WSSV). *Diseases of Aquatic Organisms* 63 (2-3): 107-111.
- Vinod M.G., Shivu M.M., Umesh K.R., Rajeeva B.C., Karunasagar I. & Karunasagar I. (2006) Isolation of *Vibrio harveyi* bacteriophages with a potential for biocontrol of this pathogen in hatchery environment. *Aquaculture* 255: 117–124.

W

- Walker P.J. & Mohan C.V. (2009) Viral disease emergence in shrimp aquaculture: origins, impacts and the effectiveness of health management strategies. *Reviews in Aquaculture* 1: 125–154.
- Walker P.J. & Winton J.R. (2010) Emerging viral diseases of fish and shrimp. *Veterinary Research* 41 (6): 51.
- Walsh P.J., Kajimura M., Mommsen T.P. & Wood C.M. (2006) Metabolic organization and effects of feeding on enzyme activities of the dogfish shark (*Squalus acanthias*) rectal gland. *Journal of Experimental Biology* 209: 2 929-2 938.
- Wang Y.B., Xu Z.R. & Xia M.S. (2005) The effectiveness of commercial probiotics in northern white shrimp *Penaeus vannamei* ponds. *Fisheries Science* 71 (5): 1 036–1 041.
- Wang Y., Bian Y.Z., Wu Q. & Chen G.Q. (2008) Evaluation of three-dimensional scaffolds made of blends of hydroxyapatite and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) for bone reconstruction. *Biomaterials* 29: 2 858-2 868.

- Wang Y., Yin J. & Chen G.Q. (2014a) Polyhydroxyalkanoates, challenges and opportunities. *Current Opinion in Biotechnology* 30: 59–65.
- Wang W., Wu X., Liu Z., Zheng H. & Cheng Y. (2014b) Insights into hepatopancreatic functions for nutrition metabolism and ovarian development in the crab *Portunus trituberculatus*. *In: Gene discovery in the comparative transcriptome of different hepatopancreas stages* (Buratti E. ed.), e84 921. *PLoS ONE* 9(1).
- Watson R. R. & Dickson K.A. (2001) Enzyme activities support the use of liver lipid-derived ketone bodies as aerobic fuels in muscle tissues of active sharks. *Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches*, 74 (2): 273-282.
- Wellen R.M.R., Canedo E.L., Lima C.A.V., Araujo I.C. Jr. & Almeida Y.M.B. (2015) The effect of polystyrene on the crystallization of poly(3- hydroxybutyrate). *Materials Research Bulletin* 18 (2): 235–239.
- Weltzien F.A., Hemre G.I., Evjemo J.O., Olsen Y. & Fyhn H.J. (2000) β -Hydroxybutyrate in developing nauplii of brine shrimp (*Artemia franciscana* K.) under feeding and non-feeding conditions. *Comparative Biochemistry and Physiology Part B* 125: 63–69.
- Whitehead K.A. & Verran J. (2009) The effect of substratum properties on the survival of attached microorganisms on inert surfaces. *In: Marine and industrial biofouling* (Flemming H.C., Murthy P.S., Venkatesan R. & Cooksey K.E. eds.), pp. 13–33, Springer, New York.

Wickins J.F. (1976) The tolerance of warm-water prawns to recirculated water. *Aquaculture* 9: 19-37.

Wickins J.F. & Lee D.O.C. (1992) *Crustacean Farming. Ranching and Culture* (Second Edition). Blackwell Science, 446 p.

Wouters R., Van Hauwaert A., Naessens E., Ramos X., Pedrazzoli A. & Lavens P. (1997) The effect of dietary n-3 HUFA and 22:6n-3/20:5n-3 ratio on white shrimp larvae and postlarvae. *Aquaculture International* 5: 113–126.

Wouters R., Lavens P., Nieto J. & Sorgeloos P. (2001) Penaeid shrimp broodstock nutrition: an updated review on research and development. *Aquaculture* 202: 1–21.

X

Xu X.L, Ji W.J, Castell J.D & O'Dor R.K (1994) Essential fatty acid requirement of the Chinese prawn, *Penaeus chinensis* *aquaculture* 127: 29–40.

Y

Yang C.C., Chen S.N., Lu C.L., Chen S., Lai K.C. & Liao W.L. (2014) Effect of mushroom beta glucan (MBG) on immune and haemocyte response in pacific white shrimp (*Litopenaeus vannamei*). *Journal of Aquaculture Research and Development* 5: 275.

Yousefian M. & Amiri M.S. (2009) Review of the use of prebiotic in aquaculture for fish and shrimp. *African Journal of Biotechnology* 8: 7 313–7 318.

Yue F., Pan L., Xie P., Zheng D. & Li J. (2010) Immune responses and expression of immune-related genes in swimming crab *Portunus trituberculatus* exposed to elevated ambient ammonia-N stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 157: 246–251.

Z

Zammit V.A. & Newsholme E.A. (1979) Activities of enzymes of fat and ketone-body metabolism and effects of starvation on blood concentrations of glucose and fat fuels in teleost and elasmobranch fish. *Biochemical Journal* 184 (2): 313-322.

Zhang B., Lin W., Huang J., Wang Y. & Xu R. (2010) Effects of artificial substrates on the growth, survival and spatial distribution of *Litopenaeus vannamei* in the intensive culture condition. *Iranian Journal of Fisheries Sciences* 9 (2): 293-304.

Zhang B. (2011) Influence of the artificial substrates on the attachment behavior of *Litopenaeus vannamei* in the intensive culture condition. *International Journal of Animal and Veterinary Advances* 3 (1): 37-43.

Zhang J., Chen L., Dong H., Duan Y., Li Z., Wen G., Chen J., Feng Z., Xu W. & Xie J. (2016) Artificial substrates in zero-water-exchange culture system regulate the rearing performance of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) under the winter indoor condition. *Aquaculture Research* 47 (1): 91–100.

- Ziemann D.A., Walsh W.A., Saphore E.G. & Fulton-Bennet K. (1992) A survey of water quality characteristics of effluent from Hawaiian aquaculture facilities. *Journal of World Aquaculture Society* 23: 180–191.
- Zin K.Y. & Chu C.J. (1991) Acute toxicity of ammonia to *Penaeus japonicus* Bate juveniles. *Aquaculture Research* 22: 259–263.
- Zinn M., Witholt B., Egli T. (2001) Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Advance Drug Delivery Reviews* 53: 5–21.

SUMMARY

The potential beneficial effects of PHB have been tested in various aquatic farmed species. Growth promotion, improved robustness against adverse environmental conditions and improved resistance against pathogenic bacteria are few of the documented beneficial effects of PHB. In addition, PHB was also reported to serve as energy source and found to affect lipid metabolism in aquatic fish species. Despite the current reports on the beneficial effects of PHB in aquatic farmed species, its actual mechanisms of action remained largely unexplored. There are, also, very limited attempts made to explore the potential beneficial effects of PHB on penaeid shrimp. Hence, the aim of this PhD research.

The supplementation of crystalline PHB (98 % poly- β -hydroxybutyrate - 2 % poly- β -hydroxyvalerate) at 1 g L⁻¹ via *Artemia* in a short term enrichment strategy significantly increased the survival of postlarval tiger shrimp *Penaeus monodon*. Improved robustness and resistance of the postlarvae against adverse environmental condition and pathogenic bacteria were also observed. The short-term co-supplementation with lipid emulsion rich in highly unsaturated fatty acids (HUFAs) at 0.3 g L⁻¹ did not add to these effects (Chapter 2). In attempt to explain the survival increasing effect of PHB, the influence of dietary PHB supplied either in crystalline or amorphous form (lyophilized cells of *Ralstonia eutropha* DSM545 containing 75 % of PHB on dry weight) on the lipid contents in crustaceans using *Artemia* sp. as a model species was explored in Chapter 3. Dietary PHB supplemented at 100 mg L⁻¹ for 48 h significantly increased the total lipid contents from 6.5 \pm 0.2 % on DW in starved *Artemia* to 8.3 \pm 0.5 % and 9.3 \pm 0.7 % on DW in *Artemia* supplied with crystalline and amorphous PHB, respectively. Based on a carbon isotope tracer study, PHB offered in natural matrix to postlarval whiteleg shrimp *Litopenaeus vannamei* is not used

as building block but probably rather as energy source (Chapter 4). A completely new approach to use PHB in penaeid shrimp culture, namely the use of PHB-based bioplastics as substratum in penaeid culture systems, was explored in Chapter 5. The survival of postlarval tiger shrimp provided with PHB substratum made out of PHB type DP9002 (Metabolix GmbH, Köln, Germany) was $88.7 \pm 3.4 \%$ and this was significantly higher as compared to postlarvae provided conventional substratum consisting of polyvinylchloride (PVC) pipes ($67.3 \pm 6.5 \%$). In a separate experiment, provision of PHB substratum made out of PHB type P209 (Biomer, Krailling, Germany) to postlarval whiteleg shrimp *L. vannamei* (106 ± 52 mg) also resulted in significantly higher survival of $83.6 \pm 3.4 \%$ as compared to $73.9 \pm 3.0 \%$ for postlarvae provided with PVC substratum. A trend of improved robustness against adverse environmental conditions and increased resistance to pathogenic *Vibrio* was also observed in postlarval tiger shrimp provided with PHB substratum as compared to postlarvae provided with PVC substratum. The additional test conducted to evaluate the effect of PHB substratum on the water quality resulted in a trend towards higher total ammonia-nitrogen (TAN) conversion from an initial concentration of 1.75 ± 0.0 mg L⁻¹ to 0.35 ± 0.04 mg L⁻¹ after 72 h while using no substratum and PVC substratum lead to TAN concentrations of 1.28 ± 0.06 mg L⁻¹ and 1.23 ± 0.10 mg L⁻¹, respectively. Moreover, the level of total inorganic nitrogen (TIN) after 72 h was significantly lower for PHB substratum (1.25 ± 0.27 mg L⁻¹) as compared to either no substratum (2.17 ± 0.18 mg L⁻¹) or PVC substratum (2.27 ± 0.19 mg L⁻¹).

Overall, the findings obtained in this PhD research have pointed out the important contributions of the bacterial storage compound PHB in improving the survival and overall

performance of penaeid shrimp. Future studies are needed to determine whether PHB, once ingested and digested in the gut of penaeid shrimp, is degraded into β -HB monomer and to determine whether this product could affect gut-associated microbial biota. Further study is also needed to evaluate the mechanisms behind the total ammonia-nitrogen removal capacity of PHB when offered as artificial substratum. Finally, strategies to lower costs of PHB are foreseen to be essential in order to render PHB more useful in the future.

SAMENVATTING

Het potentieel positief effect van PHB werd reeds getest op aquatisch soorten. Groei promotie, verbeterde weerstand tegen nadelige omgevingscondities en verbeterde weerstand tegen pathogene bacterien zijn enkele van de gedocumenteerde positieve effecten van PHB. Bovendien kan PHB ook dienen als een energiebron en heeft het een effect op het vetmetabolisme van aquatische vissoorten. Deze rapporten hebben betrekking op vissoorten terwijl de applicatie in penaeide garnalen nog niet werd uitgetest. Dit vormt het onderwerp van dit doctoraat.

De toevoeging van kristallijn PHB (98 % poly- β -hydroxybutyrate - 2 % poly- β -hydroxyvalerate) aan 1 g L^{-1} via *Artemia* in een korte aanrijking verhoogde de overleving van postlarvale *Penaeus monodon* op significante wijze. Verhoogde weerstand en robuustheid van de post-larven tegen nadelige omgevingscondities en pathogene bacterien werd ook opgetekend. Toevoeging over een korte periode van vetemulsies rijk aan polyonverzadigde vetzuren aan 0.3 g L^{-1} had geen effect (Hfst 2). In een poging om het overlevingseffect te verklaren, werd een proef opgezet waarin kristallijn zowel als amorf PHB (gevriesdroogde cellen van *Ralstonia eutropha* DSM545 met 75 % PHB op drooggewicht) werd toegevoegd aan gnotobiotische *Artemia* en het effect op vetgehalte bepaald (Hfst 3). PHB toegevoegd aan het dieet aan 100 mg L^{-1} voor 48 uur verhoogde het vetgehalte van $6.5 \pm 0.2 \%$ op drooggewicht in uithongerende *Artemia* tot $8.3 \pm 0.5 \%$ tot $9.3 \pm 0.7 \%$ door kristallijn of amorf PHB. Door een proef uitgevoerd met koolstof isotoop tracer, kon aangetoond worden dat PHB in zijn natuurlijke matrix (bacteriele cellen) niet gebruikt wordt als een bouwstof voor cellen van penaeide garnalen, maar eerder als een energiebron (Hfst 4).

Een nieuwe benadering voor PHB gebruik, namelijk het gebruik van bioplastiek-gebaseerde PHB als substratum, werd uitgetest in Hfst 5. De overleving van postlarven van tijgergarnaal met PHB als substratum (type DP9002; Metabolix GmbH, Köln, Germany) was $88.7 \pm 3.4 \%$. Dit was significant hoger vergeleken met de overleving van postlarven die een conventioneel substratum (polyvinylchloride (PVC) pijpen) werden aangeboden ($67.3 \pm 6.5 \%$). In een andere proef, de aanwezigheid van PHB substratum gemaakt van PHB type P209 (Biomer, Krailling, Germany), resulteerde ook in een significante verhoging van de overleving van postlarven van de garnaal *Litopenaeus vannamei* (106 ± 52 mg) $83.6 \pm 3.4 \%$, vergeleken met $73.9 \pm 3.0 \%$ voor postlarven die een PVC substratum werden aangeboden. Een trend tot een verhoogde robuustheid tegen nadelige omgevingcondities en verhoogde resistentie tegen pathogene *Vibrio* werd ook waargenomen in postoven van tijgergarnalen door PHB substratum. In een andere test uitgevoerd om het effect van PHB substratum op waterkwaliteit te bestuderen, een trend tot hogere totaal ammonium stikstof (TAN) omzetting werd waargenomen (van 1.75 ± 0.0 mg L⁻¹ tot 0.35 ± 0.04 mg L⁻¹ na 72 h contact met PHB substratum) in verlijking met contact met PVC substratum (2.27 ± 0.19 mg L⁻¹). of geen substratum (2.17 ± 0.18 mg L⁻¹)¹⁾

In het algemeen werd aangetoond in dit doctoraat dat PHB een belangrijke bijdrage kan leveren tot een verbetering van de overleving en prestatie van penaeide garnalen. Toekomstige studies zouden kunnen focussen op de vraag of opgenomen PHB, door afbraak in het verteringskanaal van garnalen tot monomeren, de samenstelling van de microbiele gemeenschap kan beïnvloeden. Er is ook verder onderzoek nodig naar het

mechanisme achter de verwijdering van TAN door PHB artificiele substrata. Tenslotte moeten er strategieën ontwikkeld worden om de kost van PHB te verlagen om op dit manier de toepassing ervan in aquacultuur te bevorderen.

APPENDICES

APPENDIX 1: CURRICULUM VITAE

GLADYS LUDEVESE-PASCUAL

PERSONAL INFORMATION

Date of Birth	November 23, 1984
Place of Birth	Danao, Bohol, Philippines
Nationality	Filipino
Gender	Female
Marital Status	Married

EDUCATIONAL BACKGROUND

Doctor of Philosophy in Applied Biological Sciences
Laboratory of Aquaculture and *Artemia* Reference Centre
Ghent University, Belgium
October 2011-2016

Master of Science in Aquaculture (2007-2009)
Laboratory of Aquaculture and *Artemia* Reference Centre
Ghent University, Belgium
Graduated with Great Distinction

Bachelor of Science in Biology Major in Marine Biology (2001-2005)
Leyte State University (now Visayas State University)
Baybay City, Leyte, Philippines
Graduated *Cum Laude*

Secondary Education (1997 – 2001)
Ubay National Science High School
Fatima, Ubay, Bohol, Philippines
Graduated with consistent honors

EMPLOYMENT HISTORY

Associate Researcher (May 2010-December 2011)
Southeast Asian Fisheries Development Centre/Aquaculture Department
(SEAFDEC/AQD) Tigbauan, Iloilo 5021
Philippines

Task(s):

- a) Carried out mass production techniques for the marine thraustochytrid, *Schizochytrium* sp. LEY7 as alternative source of lipid and polyunsaturated fatty acids (PUFAs) for aquaculture species.

Research Assistant I (Nov 2005-Sept 2007)

Southeast Asian Fisheries Development Centre/Aquaculture Department
(SEAFDEC/AQD) Tigbauan, Iloilo 5021
Philippines

Task(s):

- a) Assisted in the conduct of behavioural conditioning experiment for hatchery-grown Donkey's ear abalone *Haliotis asinina*;
- b) Culturing and general management of ornamental marine species (*Hippocampus* sp.).

Research Aide (Aug 2005-Nov 2005)

Institute of Tropical Ecology
Visayas State University (formerly Leyte State University)
Baybay City, Leyte 6521-A
Philippines

Task(s):

- a) Field survey of marine turtle nesting sites.

GRANTS RECEIVED

- Ghent University - Doctoral School funding for participation to Doctoral Training Programme Specialist course on Algal biodiversity – culturing, preservation and characterization of micro- and macroalgae for basic and applied research held in Ghent University, Ghent Belgium, from Sept. 11-19, 2015.
- Asian Pacific Chapter-World Aquaculture Society (APC-WAS) travel grant (Oral presenter during the World Aquaculture 2015 held in Jeju Island South Korea from May 26-30, 2015).
- Vlaamse Interuniversitaire Raad (VLIR) scholarship grant (Doctor of Philosophy in Applied Biological Sciences, Ghent University Belgium, 2011-2016)
- Vlaamse Interuniversitaire Raad (VLIR) scholarship grant (Master of Science in Aquaculture, Ghent University Belgium, 2007-2009)
- Vlaamse Interuniversitaire Raad (VLIR) Internship grant (Exchange Student, Research Institute of Aquaculture No.1 Vietnam, July 7-August 17, 2008)
- Vlaamse Interuniversitaire Raad (VLIR) South-South Mobility Program (Exchange Student, Ocean University of China, Qingdao China, October 22 - December 22, 2009)

PAPERS SUBMITTED FOR COMPLETION OF DEGREE/PUBLICATIONS

Artificial Reefs in the coastal waters of Leyte State University: Epibiontic and fish communities after ten years of deployment (Bachelor's Thesis)

Stable isotope and mineral profile as an estimate of prey preference in two coral species *Pinnigorgia* sp. and *Stylophora pistillata* grown in captivity (Master's Thesis)

Ludevese-Pascual G., Laranja J.L.Q., Amar E.C., Sorgeloos P., Bossier P. & De Schryver P. (2016) Poly-beta-hydroxybutyrate-enriched *Artemia* sp. for giant tiger prawn *Penaeus monodon* larviculture. Aquaculture Nutrition (early view) doi:10.1111/anu.12410.

Ludevese-Pascual G., Dela Peña M. & Tornalejo J. (2016) Biomass production, proximate composition and fatty acid profile of the local marine thraustochytrid isolate, *Schizochytrium* sp. LEY7 using low-cost substrates at optimum culture conditions. Aquaculture Research 47: 318–328. doi:10.1111/are.12494.

Laranja J.L., **Ludevese-Pascual G.L.**, Amar E.C., Sorgeloos P., Bossier P. & De Schryver P. (2014) Poly-beta-hydroxybutyrate (PHB) accumulating *Bacillus* spp. improve the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae. Veterinary Microbiology 173(3-4):310-7.

Buen-Ursua S. M. A. & **Ludevese G.** (2011). Temperature and size range for the transport of juvenile donkey's ear abalone *Haliotis asinina* Linne. Aquaculture Research 42 (8): 1206-1213.

De Schryver Peter, Bossier Peter & **Ludevese-Pascual Gladys**. Use of poly-β-hydroxybutyrate (PHB) as housing to homogenize growth and increase survival of crustaceans in aquaculture systems. Universiteit Gent (Sint-Pietersnieuwstraat 25, 9000 Gent, 9000, BE) WO/2016/146736
<http://www.sumobrain.com/patents/WO2016146736A1.html>

ORAL/POSTER PRESENTATION

Gladys Ludevese-Pascual, Farhana Ahmed, Marleen De Troch, Edgar Amar, Peter Bossier & Peter De Schryver. Stable ¹³C isotope analysis showed poly-β-hydroxybutyrate (PHB) assimilation in the brine shrimp *Artemia franciscana* as a time and diet-mediated process. Oral Presentation. World Aquaculture Society (WAS)-Asian-Pacific Aquaculture (WAS-APC) 2015, Jeju Island South Korea, May 26-30, 2015.

Gladys Ludevese-Pascual, Edgar Amar, Peter Bossier & Peter De Schryver. Poly- β -hydroxybutyrate (PHB) supplementation influences the lipid levels and fatty acid composition of axenic *Artemia*. Poster presentation. VLIZ Young Marine Scientists' Day, Vives Brugge, Feb. 20, 2015.

Gladys Ludevese-Pascual, Milagros Dela Peña, Jilla Tornalejo & Pet Anthony Pascual. Low-cost production of the marine thraustochytrid isolate *Schizochytrium* sp. LEY7 as alternative source of docosahexaenoic acid (DHA) important to humans and aquaculture. Poster Presentation. Regional Health Research Summit 2014, Leyte Normal University (LNU), Tacloban City Philippines (3rd placer).

Gladys Ludevese-Pascual, Milagros Dela Peña, Ofelia Reyes & Jilla Tornalejo. Low-cost production of the marine thraustochytrid isolate *Schizochytrium* sp. LEY7 as larval live feed enrichment for the mangrove snapper, *Lutjanus* sp. Poster Presentation. LARVI'2013-Fish and Shellfish Symposium, Ghent University, Ghent Belgium, Sept. 2-5, 2013.

Gladys Ludevese-Pascual, Peter de Schryver, Peter Bossier, Patrick Sorgeloos, Joseph Laranja & Edgar Amar. Poly- β -hydroxybutyrate (PHB) enriched *Artemia* sp. for the giant tiger prawn *Penaeus monodon* larviculture. Oral Presentation. World Aquaculture Society (WAS)-Asian-Pacific Aquaculture (WAS-APC) 2013, Ho Chi Minh Vietnam, Dec. 2013.

G.P.J. Janssens, **G. Ludevese** and D. Petersen. Species-dependent mineral accumulation in corals. European Zoo Nutrition Congress in January 2010.

G.P.J. Janssens, **G. Ludevese**, D. Petersen and P. Boeckx. Identifying feeding behaviour of corals through stable isotope analysis. European Zoo Nutrition Congress in January 2010.

PROFESSIONAL MEMBERSHIP

- Student member, Asian-Pacific Chapter-World Aquaculture Society (APC-WAS);
- Professional Association of Diving Instructors (PADI)-Open Water SCUBA Diver; and
- Journal Club, SEAFDEC/AQD, Iloilo Philippines

APPENDIX 2: ACKNOWLEDGEMENT

“Ingratitude to man is ingratitude to God”

-Samuel ibn Naghrela (Jewish scholar and poet)-

It is my utmost joy of taking this opportunity to express my gratitude to all who have contributed to the success of this PhD work.

I would like to thank Prof. dr. ir. Peter Bossier for his expert advice and excellent mentoring throughout this difficult project, as well as Dr. ir. Peter De Schryver, without whose never-failing sympathy and encouragement, this book would have not been realized. Credit is due to Prof. Em. Patrick Sorgeloos who helped me in sourcing funds to start this PhD project. I also want to thank Dr. Joebert Toledo, Dr. Edgar Amar and Dr. Felix Ayson for their extraordinary assistance during the conduct of my doctoral research in SEAFDEC/AQD. My gratitude also goes to the VLIR-UOS and its team, especially Micheline D’ Hooge, Helke Baeyens and Julie Poppe, whose financial support made this project possible.

I would also like to thank the members of the dissertation committee: Prof. dr. ir. Geert Janssens, Prof. dr. ir. Marc Verdegem, Prof. dr. ir. Mieke Uyttendaele, Prof. Em. Patrick Sorgeloos and Prof. dr. ir. Monica Höfte for generously offering their time in reviewing and providing their constructive criticism, as well as to all co-authors, who have been greatly involved in the conceptualization in one or two chapters in this dissertation. I am also indebted to the staff of the Laboratory of Aquaculture & *Artemia* Reference Center (ARC), for allowing me to use their facilities required for culturing livefeed and their assistance during the preparation and setting up of feeding trials so as the analysis of my experimental samples. Their substantial help in preparing this manuscript is greatly appreciated. I also extend my gratitude to the staff of the Department of Biology as well as to the staff of the Isotope Bioscience Laboratory of Ghent University for accommodating my request for sample preparation and extraction on such a short notice. It is also rewarding to have known and worked with my fellow doctoral students in ARC. Their exemplary dedication for research is truly commendable.

I must also make mention of the generosity bestowed upon me by the staff of the Groot Vleehuis and the ImaQua. My confidence is soaring, thanks to their positive affirmations on every service I have rendered. The friendship and unwavering support I gained from fellow Filipino scholars and the whole Filipino community in Ghent made my stay most memorable. I had the privilege of understanding a variety of culture and beliefs, thanks to the staff of OBSG, who were never tired of organizing multicultural events. To the circle of friends I accumulated through years of staying abroad, I am well-privileged to have known you all. I am also amazed by the unfaltering prayer and spiritual support I got from the International Community Church in Ghent (ICCG), lead by Pastor Leo Proot. Entrusting me with the church music ministry gave an opportunity for me to bring honor to the Lord Jesus Christ. Our short meetings constantly reminded me that every day is a gift, not meant to fulfill our fleeting personal desires but rather, to reflect the love of our gracious Saviour Jesus Christ, who died to redeem us from our sins.

I am deeply grateful to my parents and my siblings, were it not for their unceasing prayer and constant reminder of love, I may have wandered down pointless paths and could have not the strength to face disappointments and hardships. I am also extremely indebted to my parents-in-law who, from the very beginning, were already willing and available to assume parental guidance with my 3-year old daughter amidst their physical limitations.

No amount of words could express how thankful I am for having been blessed with a husband who tried his very best not only to be a good provider but most importantly, to be both a mom and a dad to our daughter. It is also very comforting and relaxing to relate with him my scientific ideas and interests since we share the same insatiable passion for research and development. Our agreement to work on our ever progressing tummy is one thing I looked forward to. It is my genuine prayer that God will reward the kindness of your heart. I love you with the love of our Lord Jesus Christ.

My daughter Ingine, I know you were never too young to understand that I was not around when you first learned your ABC's and 123's. But I want you to know that you

have been my inspiration and as the song goes “God’s grace remain and guide you through”. Let this be my prayer for you, Anak.

To God be the Glory!

*Gladys
August 12, 2016
3:00 A.M.
Ghent University*